

Descending Controls and Peripheral Contribution to Pain in the Monosodium Iodoacetate Model of Osteoarthritis

By Louisa Townson

A thesis submitted to University College London for the degree of
Doctor of Philosophy

Supervised by: Prof. Anthony H. Dickenson

Department of Neuroscience, Physiology & Pharmacology University College
London, Gower Street,
London, WC1E 6BT

I, Louisa Danielle Townson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis sets out to elucidate whether, in a lower dose MIA model of osteoarthritis (OA), the previously observed adaptations in descending noradrenaline and serotonin (5hydroxytrptamine, 5HT) are present. We similarly aimed to ascertain whether these adaptations were reflected in the firing patterns of ON cells in the Rostral Ventromedial Medulla (RVM), as has been seen in other chronic pain models. Finally, the role of Nav1.8 containing peripheral sensory afferents and a subset of mechanically sensitive TRPC ion channels were assessed by modeling osteoarthritis (OA), using a 0.5mg dose of MIA, in knock-out and genetically manipulated mouse lines.

in vivo electrophysiology was performed in rats following the injection of 1mg MIA to the left knee to induce OA. Following behavioural assessment, single unit recordings compared the effects of spinal ondansetron or spinal atipamezole on evoked responses of dorsal horn neurones to electrical, mechanical and thermal stimuli in MIA or control rats under isoflurane anaesthesia. Separately, single-unit recordings characterized the response of ON cells in the RVM to a range of ipsilateral and contralateral mechanical stimulation to the areas of primary and secondary hypersensitivity in MIA or sham animals.

Given the absence of effect of either drug on evoked responses of dorsal horn neurones during the 1mg MIA model, it seems possible that modifications to the extent of descending facilitation or inhibition during the MIA model are dose and time dependent. Recordings in the brainstem, the majority of which appear to be taken from the NGC, exhibited no significant adaptations, further suggesting a lack of recruitment of descending control in this 1mg MIA model. This work adds to a growing body of evidence suggesting that the descending control of pain is highly adaptive, depending upon both the extent and profile of the insult during the initiation and maintenance of chronic pain conditions.

No clear or significant sparing effect was observed through the KO of either TRPC3, TRPC6, the double TRPC3/6 KO, or through the sensory ablation of Nav1.8 containing neurones. This may indicated a role for redundancy within the mechano-sensory system of the periphery, rendering these channels poor targets of analgesia in OA.

Acknowledgements

I'd like to dedicate this thesis to the memory of my wonderful stepfather, Mark. While I wish you could be here to see me stagger across the finish line, I am so glad you got to see me start along this path. It's entirely thanks to your love, faith and guidance that I am here today, and I will never forget that. I hope I made you proud.

To Tony, I will always be grateful for the opportunity to be part of the infamous House of Pain. These have been the best years of my life. While the science and the mentoring have been next to none, it will always be the support and friendship I have found in this lab which will stay with me. Thanks for the advice, the stories and the 4pm lab drinks to celebrate the sun coming out!

Especial thanks go to Matt and Ryan, for your patience teaching me e-phys; to Kirsty and Leonor, for always having the best advice; and to Jess, for knowing exactly when I needed to let off steam – be that over coffee, a night of shots with the lads or ANNUALSKI. It really has been a privileged and a pleasure to work along side you all – Wahida, Lucy, Sital, Skip, Kirsty, Leonor, Matt, Liam, Jess, Ryan, Lauren, Stevie and Tom.

The same goes for Mike and Jane- thanks for all you taught me. To John, I am hugely grateful for the opportunity to expand my horizons, meet the mice and experience your tireless enthusiasm for science.

I want to thank my Mum for being my rock – there for every triumph (*"I actually found a cell today Mum!"*) and every moment I lost faith in myself, or the science. You truly do have a PhD in motherhood.

To everyone who listened to me fret or just helped distract me from my worries with a big glass of wine – particularly my sister, the OHS girls and the A team – thank you for keeping me sane.

But most of all, Cam. Thank you for being my very own Captain Awesome. You've shown incredible patience and understanding, never begrudging me another sunny Saturday in the flat writing up. This is just another of many finish lines I hope to cross with you at my side.

Abbreviations

5HT	Serotonin
AC	Adenylate Cyclase
ACC	Anterior Cingulate Cortex
Ach	Acetylcholine
AMY	Amygdala
ANOVA	Analysis Of Variances
AP	Action Potential
ASICs	Acid Sensing Ion Channels
ATP	Adenosine Triphosphate
AUC	Area Under The Curve
BDNF	Brain Derived Neurotropic Factor
BF	Biceps Femoralis
BK	Bradykinin
BMI	Body Mass Index
BSU	Biological Services Unit
C-LTM	C Low Threshold Mechanoreceptors
CAMKII	Calcium Calmodulin Dependent Protein Kinase II
cAMP	Cyclic Adenosine Monophosphate
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin Gene Related Peptide
CIP	Congenital Insensitivity To Pain
CL	Contralateral
CMH	C Mechano-Heat Sensitive
CNS	Central Nervous System
COX	Cyclooxygenase
CPP	Conditioned Place Preference
CPPD	Calcium Pyrophosphate Dehydrate
CRD	Colorectal Distension
CREB	CAMP Responsive Element
CS	Central Sensitisation
CVD	Cardiovascular Disease
DKO	Double Knock Out
DLP	Dorsolateral Pontine
DMH	Dorsomedial Nucleus Of The Hypothalamus
DN4	Douleur Neuropathique In 4 Questions
DNIC	Diffuse Noxious Inhibitory Controls
DRG	Dorsal Root Ganglion
DTA	Diphtheria Toxin A
EMA	European Medicines Agency
EMG	Electromyography
EPSP	Excitatory Post Synaptic Potential

ERK	Extracellular Signal Regulated Kinase
ES	Electrical Stimulation
EXIN	Excitatory Interneurons
FDA	Food and Drugs Agency (USA)
FRAP	Fluoride Resistant Acid Phosphatase
GABA	Gamma Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GADPH	Glyceraldehyde 3 Phosphate Dehydrogenase
GDC	Guideline Development Group
GDNF	Glial Cell Derived Growth Factor
GFRalpha1-4	GDNF Family Receptor Alpha 1-4
GI	Gastro Intestinal
GlyT2	Glycine Transporter 2
GP	General Practitioner
GPCR	G Protein Coupled Receptor
GRIP	Glutamate Receptor Interacting Protein
H+	Hydrogen Ion
IA	Intra Articular
IB4	Isolectin B4
IBS	Irritable Bowel Syndrome
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Ipsilateral
IL1beta	Interleukin 1 Beta
IL6	Interleukin 6
IL8	Interleukin 8
ININ	Inhibitory Interneuron
IT	Intrathecal
JAK	Janus Kinase
K+	Potassium Ion
KO	Knock Out
LC	Locus Coeruleus
LGIC	Ligand Gated Ion Channel
LPB	Lateral Parabrachial
LTM	Low Threshold Mechanosensing
LTP	Long Term Potentiation
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MIA	Monosodium Iodoacetate
MMP	Matric Metalloproteinase
MPC	Medial Prefrontal Cortex
Mrgpr	MAS-related G protein-coupled receptors
MRI	Magnetic Resonance Imaging

NA	Noradrenaline
NGC	Nucleus Gigantocellularis (Also known as RGC and Gi)
NGCalpha	Nucleus Gigantocellularis pars alpha
NGF	Nerve Growth Factor
NHS	National Health Service (UK)
NICE	National Institute For Clinical Excellence
NKA	Neurokinin A
NO	Nitrous Oxide
NOS	Nitric Oxide Synthase
NPY	Neuropeptide Y
NRM	Nucleus Raphe Magnus
NS	Nociceptive Specific Cells
NSAID	Non Steroidal Anti-Inflammatory Drug
NT-3/4	Neurotensin 3/4
NTS	Nucleus of the solitary tract
OA	Osteoarthritis
PAG	Periaqueductal Grey
PB	Parabrachial Area
PCR	Polymerase Chain Reaction
PD	Post Discharge
PEPD	Paroxysmal Extreme Pain Disorder
PEphys	Peripheral Electrophysiology
PGE2	Prostaglandin E2
PHN	Postherpetic Neuralgia
PI3k	Phosphoinositide 3 Kinase
PKA	Phosphokinase A
PKC	Phosphokinase C
PLC	Phospholipase C
Po	Posterior group thalamic nuclei
PPT	Pressure Pain Threshold
PSTH	Post Stimulus Time Histogram
PWL	Paw Withdrawal Latency
PWT	Paw Withdrawal Threshold
QST	Quantitative Sensory Testing
RA	Rheumatoid Arthritis
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RPOA	Rapidly Progressing OA
RTK	Receptor Tyrosine Kinase
RVM	Rostral Ventromedial Medulla
SAP	Saporin
SD	Sprague Dawley
SEM	Standard error of the mean

shRNA	Small Hairpin RNA
SKO	Single Knock Out
SNL	Spinal Nerve Ligation
SNRI	Serotonin Noradrenaline Reuptake Inhibitor
SP	Substance P
SpEphys	Spinal Cord Electrophysiology
STT	Spinothalamic Tract
TA	Tibialis Anterior
TCA	Tricyclic Antidepressants
TENS	Transcutaneous Electrical Nerve Stimulation
THR	Total Hip Replacement
TKR	Total Knee Replacement
TNFalpha	Tumour Necrosis Factor Alpha
Tph-2	Tryptophan Hydroxylase
TRPA1	Transient Receptor Potential Subfamily A Member 1
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPV1	Transient Receptor Potential Vanilloid 1
TTX	Tetrodotoxin
VAS	Visual Analogue Scale
VEGF	Vascular Endothelial Growth Factor
vF	Von Frey Hair
VGLUT	Vesicular Glutamate Transporter
VLM	Ventrolateral Medulla
VGSC	Voltage Gated Sodium Channel
VMpo	Ventral Posterolateral Nucleus
vs.	Versus
WB	Weight Bearing
WDR	Wide Dynamic Range
WOMAC	Western Ontario And McMaster Universities OA Index
WT	Wild Type

Table of Contents

1. General Introduction

1.1 Why Study Osteoarthritis Pain?	25
1.2 Osteoarthritis	27
1.2.1 The Healthy Joint	27
1.2.2 Structural Changes in OA	28
1.2.3 Clinical Features and Pain in OA	31
1.2.4 Risk Factors	34
1.2.5 Epidemiology	36
1.3 Current Treatments of OA Pain	39
1.3.1 Pharmacology	39
1.3.2 Surgical Intervention	44
1.4 Modeling Osteoarthritis Pain	45
1.4.1 The MIA Model	45
1.4.2 Histopathology in the MIA model	46
1.4.3 Pain like behaviour in the MIA model	47
1.4.4 Electrophysiology in the MIA model	48
1.5 An Overview of Nociception	51
1.5.1 Peripheral Nociception	51
1.5.1.1 The Fibers	51
1.5.1.2 Subdivision by sensation	52
1.5.1.3 Molecular markers defining subpopulations	52
1.5.1.4 Efferent Function	55
1.5.1.5 Joint Innervation	56
1.5.1.6 Spinal Terminations of Primary Afferents	56
1.5.1.7 Modulating Influences on Primary Afferents	58
1.5.1.8 Distinct Contribution to Hyperalgesia and Allodynia	59
1.5.2 Stimulus Transduction and Transmission	62
1.5.2.1 Mechano and Thermo Transduction	62
1.5.2.2 Voltage Gated Sodium Channels	63
1.5.2.3 Ion Channels, Chemical Stimuli and Inflammatory Pain	64
1.5.3 Peripheral Sensitization	65
1.5.3.1 Activation of LGICs	66
1.5.3.2 Activation of GPCRs	67
1.5.3.3 Tyrosine Kinase Receptor Activation	68
1.5.3.4 NGF at TrkA	69
1.5.3.5 Evidence for Peripheral Sensitization in OA	70
1.5.4 The Dorsal Horn	71
1.5.4.1 Organization	71

1.5.4.1.1	Superficial Layers.....	72
1.5.4.1.2	Deep Dorsal Horn.....	74
1.5.4.2	The Synapse and Neurotransmitters involved in Transmission.....	75
1.5.4.3	Non-neuronal Contributions to Transmission.....	76
1.5.4.4	Central Sensitization.....	77
1.5.4.4.1	Mechanisms.....	77
1.5.4.4.2	Evidence in OA.....	80
1.5.5	Ascending Projections.....	81
1.5.5.1	Spinothalamic Tract.....	81
1.5.5.2	Spino-bulbo-spinal Loop.....	82
1.5.6	The Brain, Descending Controls and Pain.....	82
1.5.6.1	Key Pain Centers.....	83
1.5.6.1.1	Periaqueductal Gray.....	83
1.5.6.1.2	Rostral Ventromedial Medulla.....	84
1.5.6.1.3	Dorsolateral Pontine Nuclei.....	86
1.5.6.2	Changes to Descending Controls During Inflammation.....	87
1.6	Thesis Aims.....	91

2 **Methods**

2.1	Animals.....	93
2.1.1	Rats.....	94
2.1.2	Mice.....	94
2.2	Induction of OA.....	95
2.2.1	Rats.....	95
2.2.2	Mice.....	95
2.3	Behaviour.....	96
2.3.1	Assessment Days.....	96
2.3.2	Acclimatization.....	96
2.3.3	Mechanical Hypersensitivity.....	96
2.3.4	Cooling Hypersensitivity.....	99
2.4	Spinal Cord Electrophysiology.....	101
2.4.1	Animal Preparation.....	101
2.4.2	Single Cell Recordings.....	103
2.4.3	Pharmacology.....	106
2.5	Electrophysiological Recordings from the RVM.....	107
2.5.1	Animal Preparation.....	107
2.5.2	Single Cell Recording.....	107
2.5.3	Experimental Protocol.....	109
2.5.4	Data Extraction.....	110

2.6 Analysis.....	112
-------------------	-----

3 Modeling Osteoarthritis Pain

3.1.1 The Importance of dose.....	113
3.1.1.1 The 1mg Model: a Reliable and Robust Model of OA Pain.....	114
3.1.1.2 Neuropathic profile of 2mg Rats.....	116
3.1.1.3 Previous literature has characterized changes in descending controls in ta 2mg MIA model of OA Pain.....	118
3.1.2 Chapter Aims.....	119
3.2 Methods.....	121
3.2.1 Animals.....	121
3.2.2 Induction of the Model.....	121
3.2.3 Behaviour Assessment.....	121
3.2.4 Electrophysiological Assessment.....	122
3.2.5 Data Analysis.....	122
3.3 Results.....	123
3.3.1 Behavioural Hypersensitivity in the MIA Model of OA.....	123
3.3.2 Spinal Neuronal Hypersensitivity in the MIA Model of OA.....	125
3.4 Discussion.....	129
3.4.1 Behavioural Hypersensitivity.....	129
3.4.2 Sensitization Observed in Spinal Electrophysiology.....	135
3.4.3 Study Limitations.....	140
3.4.4 Overall Implications.....	142

4 Descending Controls in the MIA Model

4.1.1 Serotonin.....	143
4.1.2 Noradrenaline.....	144
4.1.3 Chapter Aims.....	146
4.2 Methods.....	149
4.2.1 Animals.....	149
4.2.2 Induction of the Model.....	149
4.2.3 Behavioural Assessment.....	149
4.2.4 Electrophysiological Assessment.....	149
4.2.5 Data Analysis.....	151
4.3 Results.....	153
4.3.1 Effect of ondansetron in Early MIA Animals.....	153
4.3.2 Effect of ondansetron in Late MIA Animals.....	153
4.3.3 Effect of atipamezole in Early MIA Animals.....	156

4.3.4	Effect of atipamezole in Late MIA Animals.....	156
4.4	Discussion.....	159
4.4.1	The 1mg MIA Model of OA Fails to Recruit Descending Serotonergic Facilitatory Systems.....	159
4.4.2	The 1mg MIA Model of OA Fails to Recruit Descending Noradrenergic Inhibitory Systems.....	165
4.4.3	Question of Central Sensitization in the MIA model.....	170
4.4.4	Study Limitations.....	174
4.4.5	Overall Implications.....	177

5 Brainstem Sensitization in Osteoarthritis Pain

5.1	Introduction.....	179
5.1.1	Heterogeneity of RVM Neurones.....	179
5.1.2	Evidence of RVM Plasticity During Pain.....	182
5.1.3	Our Understanding of Brainstem Sensitization in the OA Clinic.....	185
5.1.4	Chapter Aims.....	187
5.2	Methods.....	189
5.2.1	Animals.....	189
5.2.2	Induction of the Model.....	189
5.2.3	Behavioural Assessment.....	189
5.2.4	Electrophysiology Assessment.....	189
5.2.5	Data Analysis.....	190
5.3	Results.....	193
5.3.1	Recording Sites.....	193
5.3.2	Confirmation of Behavioural Hypersensitivity.....	195
5.3.3	Electrophysiological Recordings in MIA and Sham Rats.....	195
5.3.4	Characterization of ON Cell Responses to Somatic Stimulation.....	199
5.3.5	Reclassification and ON:ON-like Cell Analysis.....	202
5.4	Discussion.....	205
5.4.1	Implications of Recording from NGC.....	205
5.4.2	The Baseline Rate of Firing and Latency to Tail Flick in MIA Rats.....	206
5.4.3	Physiological Responses of RVM On-Cells to Mechanical Stimulation of the Paw and Knee.....	209
5.4.4	Is the MIA model sufficiently severe to evoke changes in firing?.....	211
5.4.5	Study Limitations.....	214
5.4.6	Overall Implications.....	215

6 Peripheral Contribution of Osteoarthritis Pain

6.1 The Use of Mouse Models to Identify the Contribution of Specific Proteins in Healthy and Pathological Sensation.....	217
6.1.1 Voltage Gated Sodium Channels.....	217
6.1.2 Transient Receptor Potential Channels.....	220
6.1.3 Chapter Aims.....	221
6.2 Methods.....	223
6.2.1 Animals.....	223
6.2.2 Induction of the Model.....	223
6.2.3 Behavioural Assessment.....	224
6.2.4 Data Analysis.....	224
6.3 Results.....	225
6.3.1 Behavioural Hypersensitivity in the MIA Model in WT and DTA Mice.....	225
6.3.2 Behavioural Hypersensitivity During the MIA Model in WT, SKO and DKO Mice.....	226
6.4 Discussion.....	229
6.4.1 Behavioural Hypersensitivity in a Murine Model of MIA Induced OAP.....	229
6.4.2 Preserved Punctate Hypersensitivity at Day 14 and Onwards After 0.5mg MIA in Mice Lacking Nav1.8 Neurones	232
6.4.3 The Behavioural Profile of TRPC3 and TRPC6 SKO and SKO Mice	235
6.4.4 Study Limitations.....	237
6.4.5 Overall Implications.....	240
7 General Discussion	
7.1 General Summary Of Findings.....	241
7.2 Translational Relevance to the OA Clinic.....	243
7.2.1 MIA as a Model of OA Pain–The Question of Neuropathy?.....	243
7.2.2 Manipulating Descending Controls to Achieve Analgesia In OA Patients with Neuropathic Pain Qualities.....	245
7.2.3 Viability of Nav1.8 for Analgesia in OA Pain.....	245
7.3 Methodological Considerations.....	247
7.3.1 Further considerations when using the MIA model.....	247
7.3.2 Limitations of <i>in vivo</i> Electrophysiology.....	248
7.3.3 Limitations of KO Mice for the Study of Pain Mechanisms.....	249
7.4 Further Research.....	251
7.5 Closing Remarks.....	253
8 Appendix.....	255
9 References.....	259

List of Figures

- Figure 1.1 - Synovial Joint Structures Affected in OA**
- Figure 1.2 - Sagittal inversion recovery and coronal fast spin-echo magnetic resonance images of the knee joint illustrating the features of osteoarthritis**
- Figure 1.3 - National Institute for Health and Care Excellence guidelines for the management of Osteoarthritis**
- Figure 1.4 - NICE Guidelines for the treatment of OA in adults**
- Figure 1.5 - Coronal sections of control and 0.2mg injected MIA tibia at 10weeks post injection**
- Figure 1.6 - Pie chart summarizing the subdivision of DRGs according to molecular markers**
- Figure 1.7 - Primary afferent termination in the dorsal horn**
- Figure 1.8 - Mechanisms of peripheral sensitization at the peripheral terminal of a sensory afferent**
- Figure 1.9 - Electrically evoked responses of a Lamina V WDR neuron**
- Figure1.10 - Development of Secondary Hyperalgesia following capsaicin injection, as a consequence of development central sensitization in a spinal cord neuron**
- Figure1.11 - Schematic diagram of descending controls in the rat brain**
- Figure 2.1 - Behavioural Testing For Punctate Mechanical Hypersensitivity**
- Figure 2.2 - Weight Bearing Assessment Using An Incapacitance Tester**
- Figure 2.3 - Stereotaxic Set Up Of The Rat For Spinal Electrophysiology**
- Figure 2.4 - Schematic Of The Neurolog Recording System**
- Figure 2.5 - Electrical Evoked Responses From An Example WDR Cell**
- Figure 2.6 - Naturally Evoked Responses Of Lamina V WDR Neuron**
- Figure 2.7 - Example ON- , OFF- And Neutral Cells Displaying Characteristic Change In Reflex Related Firing Following Noxious Thermal Stimulation Of The Tail**
- Figure 2.8 - Depiction Of The Process Through Which Spike 2 Waveform Data Is Sorted And Filtered To Identify Individual Cells For Extraction And Analysis**
- Figure 3.2 - Articular Histology Taken From Thakur Et Al 2012, Comparing The Relative Effect Of Saline, 1mg Or 2mg MIA On Articular Cartilage At 14days After Injection**
- Figure 3.2 - Articular Histology Taken From Ivanavicius Et Al 2007 Displaying The Time Course Of The Histopathology Of A 1mg MIA Injection**

- Figure 3.3 – Monosodium Iodoacetate Induced OA Produced Significant Punctate Mechanical Hypersensitivity And A Reduction In Ipsilateral Weight Bearing Versus Shams In Both Early And Late Stages**
- Figure 3.4- Comparison Of Electrically And Dynamic Brush Evoked Responses Of Lamina V WDR Cells In MIA And Sham Animals At Either Early Or Late Stages After Injection**
- Figure 3.5 - Comparison Of Mechanically Evoked Responses Of Lamina V WDR Cells In MIA And Sham Animals At Either Early Or Late Stages After Injection**
- Figure 3.6 - Comparison Of Thermally Evoked Responses Of Lamina V WDR Cells In MIA And Sham Animals At Either Early Or Late Stages After Injection**
- Figure 3.7 - Comparison Of Dynamic Brush Evoked Responses Of Lamina V WDR Cells In Early MIA (Red) And Early Sham (Purple) Animals Depending On The Experimental Cohort.**
- Figure 4.1 – 100µg Of Spinally Administered Ondansetron Has No Significant Effect On The Evoked Responses Of Lamina V WDR Neurons In Early MIA**
- Figure 4.2 - 100µg Of Spinally Administered Ondansetron Has No Significant Effect On The Mechanically Or Thermally Evoked Responses Of Lamina V WDR Neurons In Late MIA Or Late Sham Rats, But Significantly Inhibited The Electrically Evoked A β And A δ Counts In Sham Animals.**
- Figure 4.3 - Spinally Administered Atipamezole Has No Significant Effect On The Mechanically Or Thermally Evoked Responses Of Lamina V WDR Neurons In Early MIA Or Early Sham Rats, But Significantly Inhibited The Electrically Evoked Wind Up In MIA Animals.**
- Figure 4.4 - Spinally Administered Atipamezole Has No Significant Effect On Evoked Responses Of Lamina V WDR Neurons In Late MIA Or Late Sham Rats**
- Figure 5.1 – Pictorial Summary Of A Simplified Possible Sub-Classifications Of Neurons Descending From The RVM,**
- Figure 5.2 - A Diagrammatic Representation Of Coronal Sections Corresponding To RVM Nuclei With Recording Sites Indicated**
- Figure 5.3 – Monosodium Iodoacetate Induced OA Produced Significant Mechanical Hypersensitivity In The Ipsilateral Paw Versus Both Contralateral Paws And Sham Animal Controls**
- Figure 5.4 - ON, OFF, And Neutral Cells Display Characteristic Changes In Reflex-Related Firing Following A Noxious Somatic Stimulus**

- Figure 5.5 – The Baseline Rate Of Firing, Latency To Tail Flick Following Application Of Noxious Tail Heat, And Change In Rate Of Reflex Related Firing Of ON, OFF And Neutral Cells Did Not Significantly Differ Between MIA And Sham Animals 14-16days Following Injection.**
- Figure 5.6 – The Response Of ON-Cells To Mechanical Stimulation To The Knee And Paw Following MIA And Saline Produced Divergent Responses, With A Minority Of Cells Responding In An OFF Like Manner To Reduce The Rate Of Firing**
- Figure 5.7 – The Response Of ON-Cells To Mechanical Stimulation To The Knee And Paw 14-16 days Following Injection Of Either 1mg MIA Or Saline Produced Divergent Responses Within Groups, With A Minority Of Cells Responding In An OFF-Like Manner To Reduce The Rate Of Firing**
- Figure 5.8 – Change In The Rate Of Firing Of ON-Cells Exhibiting ON-Like Changes In The Rate Of Firing Following The Application Of Von Frey Hairs Or Knee Pinch To The Ipsilateral Or Contralateral Hind Limb 14-16days After The Injection Of 1mg MIA Or Saline**
- Figure 5.9 - The ON-Like Response Of Recorded ON-Cells To Mechanical Stimulation To The Knee And Paw 14-16days After Injection Of Either 1mg MIA Or Saline**
- Figure 6.1 – Monosodium Iodoacetate Induced OA Produced Significant Punctate Mechanical Hypersensitivity And A Reduction In Ipsilateral Weight Bearing In Both WT And DTA Mice, However Differences In The Time Course Of The Behavioural Profile Are Apparent**
- Figure 6.2 - Monosodium Iodoacetate Induced OA Produced Significant Punctate Mechanical Hypersensitivity And A Reduction In Ipsilateral Weight Bearing In Both WT, TRPC3 SKO, TRPC6 SKO And TRPC3/6 DKO Mice, With No Significant Differences In The Behavioural Profiles Of These Four Mouse Lines**

List of Tables

- Table 1.1 - Prevalence of Knee OA by age and gender in the Framingham OA study**
- Table 1.2 - Structural and neurochemical markers of DRG subpopulations**
- Table 4.1 - Evidence for central sensitization in 22 published studies using the 1mg MIA model to investigate pain in OA**
- Table 5.1 - Total Animals Used and Cells Characterized Within Each Condition**

Table 5.2 - Changes in Activity of RVM ON-cells Following the Application of 8-100g vF Hairs or Knee Pinch to the Ipsilateral or Contralateral Paw 14-16days After the Injection of MIA or Saline

Chapter 1 – General Introduction

1.1 Why Study Osteoarthritis Pain?

Pain is an intrinsically aversive and hugely subjective sensory experience. In its adaptive form pain exists as an innate warning system for potential or existing damage, but in its chronic, pathological forms it can result in a severe decline in quality of life. Such is the case in arthritis, which accounts for approx. 40% of chronic pain conditions in the UK(Breivik, Collett et al. 2006).

Of the arthritic conditions, Osteoarthritis (OA) is the most common. 8.5million individuals suffer from OA in at least one joint and 71% of them are in almost constant pain, while 1million rate their pain as unbearable(Smith 2012). A further 2 in 5 say that the analgesia they are provided is either not very effective or not at all effective(Smith 2012). This has considerable consequences for the quality of life of the patient. Pain becomes a limiting factor on the ability to function and engage in day-to-day life, with a third of sufferers retiring early and 1 in 5 giving up hobbies and leisure activities(Smith 2012). OA pain similarly leads to degeneration in mood, quality of sleep and the development of psychological comorbidities(Nicholson and Verma 2004, Power, Perruccio et al. 2005).

Pain is also a primary driver behind the ~164,000 knee and hip replacement surgeries performed on the NHS each year, costing in excess of £1billion per annum(Mancuso, Ranawat et al. 1996, Cross III, Saleh et al. 2006, Birrell 2011). In the interim, patients are reliant upon narcotic analgesics, whose side effect profiles include constipation, nausea and vomiting, where even total joint replacement fails to resolve joint pain fully in 30% of patients(Wylde, Hewlett et al. 2011).

As such, OA is not simply a considerable source of suffering and disability for millions, but also places considerable financial strain on the NHS, while OA related sick days are thought to be worth millions to the economy, making the treatment of OA of great social and economic importance. In the absence of disease modifying therapies, agents that might slow or even reverse the progression of structural pathophysiology, the second greatest unmet need in the clinical is effective and tolerable analgesia.

For a period the anti-NGF therapies, namely tanezumab, looked to provide a light at the end of the tunnel for OA patients in moderate to severe, chronic pain. The results from clinical trials were compelling(Lane, Schnitzer et al. 2010, Brown, Murphy et al. 2012, Spierings, Fidelholtz et al. 2013). However, after 2 FDA mandated clinical holds and an anticipated total of 32 trials in >15,000 patients, even once licensed tanezumab is expected to be contraindicated with NSAIDs(Balanescu, Feist et al. 2014, Schnitzer, Ekman et al. 2014), due to the risk of rapidly progressing OA (RPOA), and

restricted to those patients who have failed several lines of therapy (Phase 3 trial design: NCT02528188). To date, the exact relationship between anti-NGF therapies and NSAIDs that might provoke RPOA are still unclear and it is these limits to our present understanding of OA, the mechanisms underlying pain and their relationship to overall joint health, which continually challenge the successful development of pharmacotherapy for OA.

By developing a more comprehensive understanding of the mechanisms underlying pain in OA, the discreet progression and adaptation of physiological systems over time, and building our awareness of the limitations of the models we utilize, we may be able to identify “cleaner” targets – whose analgesia successfully outstrip the side effect burden. The work presented within has aimed to better understand the time and dose related adaptations of descending control systems in a model of OA pain in order to elucidate if and when pharmacological interferences with these systems might provide effective analgesia in OA.

1.2 Osteoarthritis

Osteoarthritis is a degenerative condition characterized by the loss of function and integrity of synovial joints, most notably of the hip and knee, though it can affect a wide range of joints, including the hands, spine and shoulder. The condition can be viewed as the shared outcome of a range of genetic, metabolic and environmental triggers, though the mechanism underlying the initiation and perpetuation of this condition are not yet fully understood.

Clinically, OA is characterized by movement-evoked pain, tenderness, joint stiffness, muscle weakness, instability and episodic inflammation. Structurally, OA is described by the loss of cartilage, bony out growths and sclerosis. The stumbling block for the early diagnosis of OA, based on these criteria, is that while joints may show radiological changes, these changes are not necessarily clinically relevant for a significant period of time, limiting reportage to GPs. Additionally, the appearance of both the pain and radiological features of OA are insidious. Notably, there is also a disparity between the extent of structural alteration and clinical outcomes in patients, which may complicate matters.

1.2.1 The Healthy Joint

A joint can be simply defined as the articular meeting point of two or more bones, largely with the aim of providing either mechanical support and/or movement of the skeleton. The true picture is slightly more complex, with three subcategories of joint based on their structure and function: Fibrous, Cartilaginous and Synovial. The synovial joint is the most common of the joints, for example the knees, hips, hands and shoulder, and is the category of joint most commonly affected by OA. A dense fibrous capsule unites these joints, where the synovial fluid held in this the synovial cavity of this capsule acts to lubricate joint articulation. Given the prevalence of OA of the knee and the methods we use to model OA (See Chapter 3), I will focus on the knee joint to demonstrate synovial joint anatomy and pathology.

As in all synovial joints, the articulating bony surfaces of the knee are not in direct contact. The articular surfaces of the femur and tibia, as well as the posterior surface of the patella, are covered by hyaline articular cartilage to cushion the joint and facilitate smooth movement. This cartilage is a dense extracellular matrix, composed largely of type II collagen, proteoglycans and water, which is secreted and maintained by the highly differentiated mesenchymal cells, known as chondrocytes. This cartilage is both a-vascular and lacking innervation.

In addition to the cartilage, the joint is also cushioned and lubricated by the fluid of the synovial cavity, the friction coefficient of which is low enough to facilitate much freer movement. This synovial fluid is crucial to the maintenance of cellular entities within the cartilage, supplying the necessary gases and nutrients for survival and continued cartilage maintenance. This fluid is secreted by the synoviocytes that line the inner of the capsule, forming the synovium. These cells are highly metabolically active, responsible for both the nourishment of the chondrocytes but also the removal of metabolites and products of matrix degradation. In contrast to the cartilage, this structure is highly vascularized and innervated.

The capsule itself is made up of fibrous connective tissue emerging from the periosteum of each bone, forming the boundaries of the joint. In other words, those areas within the capsule are designated as intra-articular. In addition to this capsule, the bones of the joint are held together by ligaments and stabilized by the surrounding muscle groups, which themselves are connected by tendons directly to the bone. This structure essentially holds the joint together and acts to limit the extent and direction of flexion in the joint.

1.2.2 Structural Changes in OA

At a macroscopic level, the characteristic change observed in OA is a loss, degradation or fibrillation of the hyaline cartilage covering the epiphyses of the synovial joint(Poole 1993). At a more microscopic/molecular level, there are also chondrocyte cell clusters, matrix depletion and composition changes(Poole 1993). Although the exact mechanisms vary hugely between individuals, the cartilage is believed to be one of the principal sites of initiation of OA. Often described as the loss of equilibrium in the joint, it is believed there is a shift of the discreet balance between synthesis and break down of this hyaline cartilage, triggering the transformations observed in OA.(Eyre 2004)

Divergence from “healthy” cartilage structure is not a uniform process. There is a huge spectrum of change; from surface irregularities and alterations in matrix composition, which can only be observed at the microscopic level; to the loss of huge swathes of cartilage to expose the subchondral bone.

However, OA is not simply a disease of the cartilage but has diverse effects within the surrounding tissues. Notably there are distinct changes in the bone crucial to the diagnosis and perpetuation of OA. These include increased density of the juxta-articular bone, subchondral trabecular bone structure modifications, osteophyte (bony outgrowth) formation and sub-articular cysts (Kellgren and Lawrence 1957, Kean, Kean et al. 2004, Wieland, Michaelis et al. 2005).

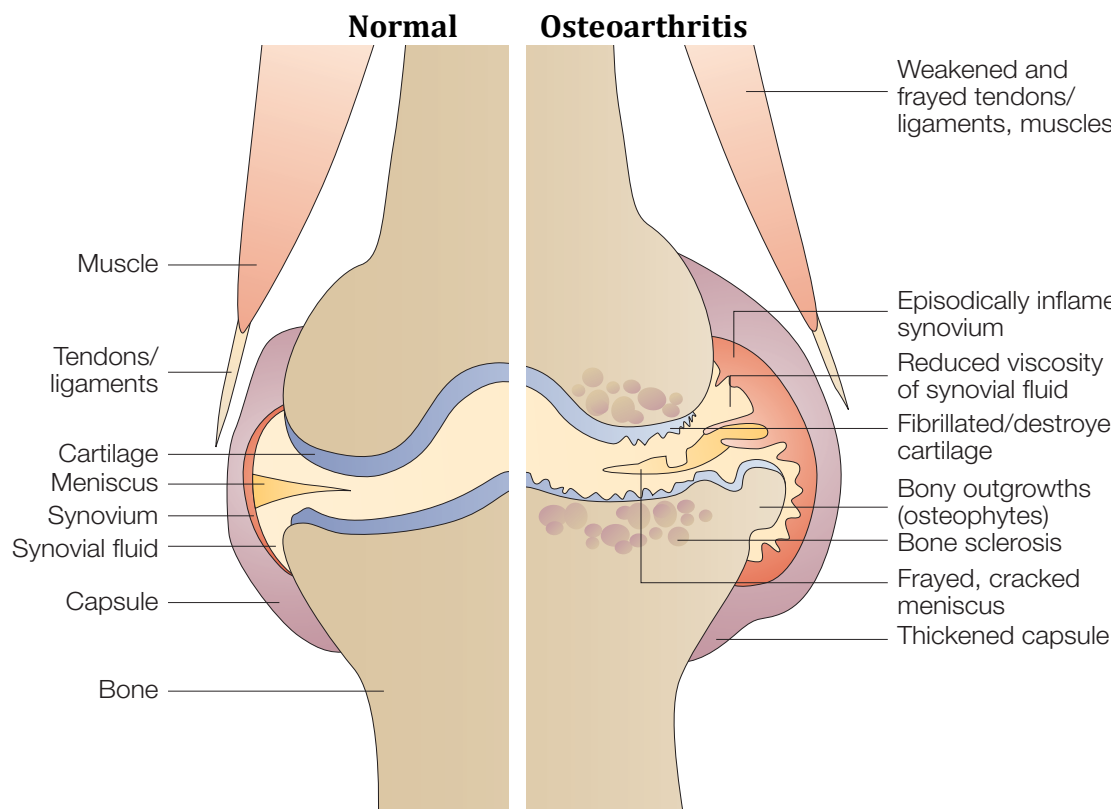


Figure 3.1: Synovial Joint Structures Affected in OA. Left| Healthy joint lacking cartilage fissures or joint inflammation. Right| OA joint - cartilage erosion/fibrillation, episodic synovitis, bone remodeling and muscle weakness. (Weiland, Michaelis et al 2005)

There is also evidence of neurovascularization of areas of bone remodeling, including infiltration into the neighboring cartilage, promoting calcification or ossification to compromise the natural barrier between the articular cartilage and subchondral bone (Ghosh and Cheras 2001, Suri, Gill et al. 2007, Walsh, Bonnet et al. 2007). Goldring and Goldring 2010 argue that an imbalance in the adaptation of the cartilage and bone undermines the discrete tissue relationships leading to pathology in OA, though it is largely believed that cartilage destruction precedes bone pathology (Felson and Neogi 2004, Goldring and Goldring 2010).

OA is also a disease of the synovium. During OA this thin cell barrier becomes both inflamed and thickened (Fernandez-Madrid, Karvonen et al. 1994, Wieland, Michaelis et al. 2005), where acute

flares are characterized by effusion, warmth and tenderness, resulting in a degree of inactivity/morning stiffness(Bonnet and Walsh 2005). This synovitis is detectable both by imaging, arthroscopy and histology.

Synovitis may be either a primary initiating factor (e.g. following injury) or secondary to other changes occurring in the joint, including the removal of matrix degradation products, such as hyaluronic acid, from the synovia or the presence of calcium pyrophosphate dehydrate (CPPD) crystals(Bonnet and Walsh 2005). This debris initiates cell-mediated immune responses, while Uric Acid released by damaged and dying cells of the joints activates the inflammasome, leading to the release of inflammatory mediators such as IL1 β , TNF- α , IL-6, IL-8, NO and PGE₂(Guerne, Terkeltaub et al. 1989, Liu, O'Connell et al. 2000, Sellam and Berenbaum 2010, Denoble, Huffman et al. 2011). It is these inflammatory factors mediating synovitis and a large degree of the pain and tenderness in OA(Sellam and Berenbaum 2010). These factors also perpetuate the changes already occurring in the joint, including fibroblast proliferations, immune cell recruitment, cellular apoptosis, angiogenesis and sensory innervation, starting a vicious cycle of OA progression(Walsh 1999, Haywood, McWilliams et al. 2003, Bonnet and Walsh 2005, Sellam and Berenbaum 2010). Despite this, OA is not classified as an inflammatory disease.

Classically, X-rays are used to assess changes in structural pathology in patients with OA. These changes are then quantified using scales which rate the radiographic pathologies observed, such as the previously described loss of joint space, osteophytes or bone sclerosis, for example the Kellgren Lawrence Grade(Kellgren and Lawrence 1957). In addition to X-ray however, Magnetic Resonance Imaging (MRI) can be used to provide enhanced perspectives on the modifications occurring in the joint as OA progresses. MRIs have the benefit of providing soft tissue contrast, allowing for the assessment of effusions, synovitis and changes in hyaline cartilage - changes that may often precede the development of radiographic changes - as well as bone marrow lesioning. Conversely however, MRIs can highlight “abnormalities” in knees rated as healthy, i.e. individuals without an OA diagnosis and scoring 0 on the Kellgren Lawrence grade, who suffer no pain at all. This was demonstrated in the Framingham Osteoarthritis Study, in which 86-88% of painless knees showed at least one type of pathology(Guermazi, Niu et al. 2012).

The difficulty with the use of structural changes to assess OA is that, at present, the relationship between these changes, disease progression and pain are poorly understood. It is not yet possible to identify a causal relationship between specific structural changes and the clinical realities of pain in OA(Hunter, Guermazi et al. 2013). As such these measures are of limited use, since they lack the sensitivity required to assess disease progression, clinical outcomes of disease modifying drugs and,

most importantly to my own research, they are poorly association with joint pain (Buchanan and Kean 2002, Finan, Buenaver et al. 2013).

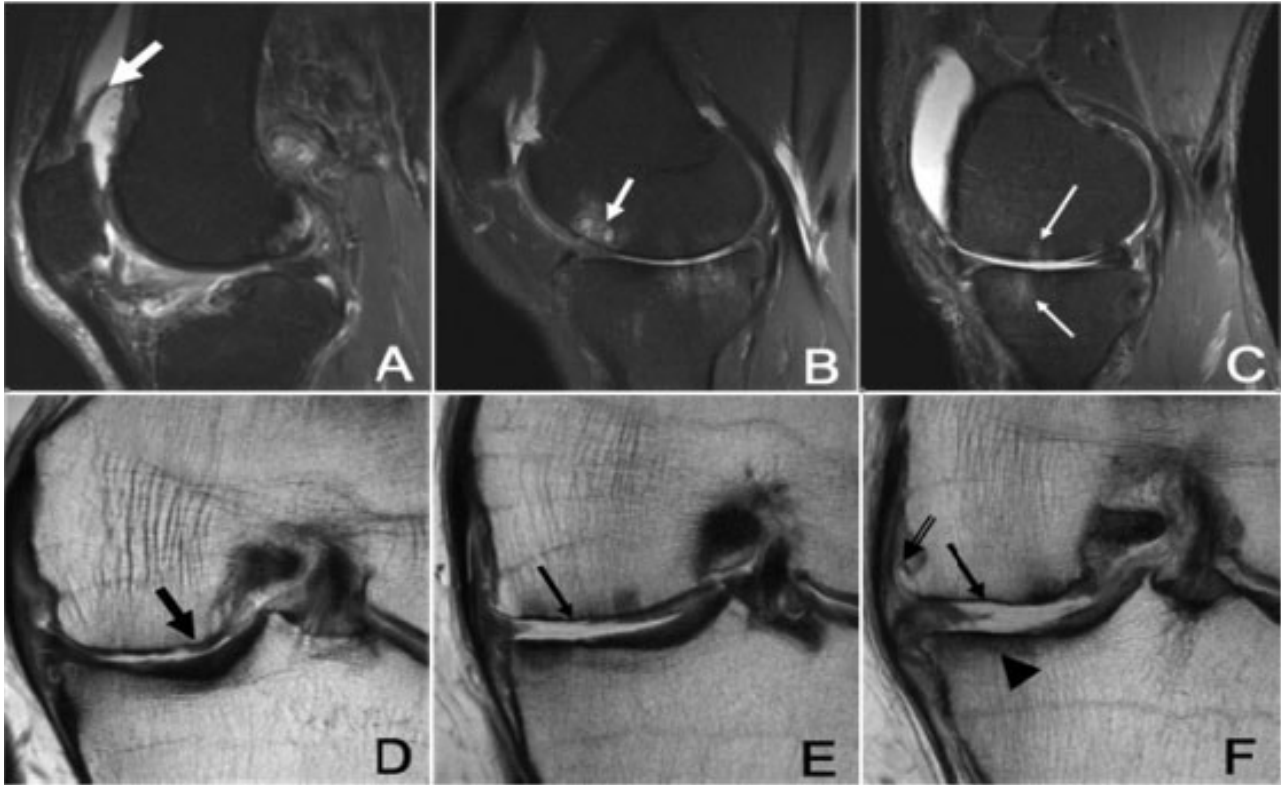


Figure 1.2: Sagittal inversion recovery (A-C) and coronal fast spin-echo (D-F) magnetic resonance images of the knee joint illustrating the features of osteoarthritis. A) reactive synovitis (arrow), B) subchondral cyst formation (arrow), C) bone marrow edema (arrows), D) partial-thickness cartilage wear (arrow), and E and F), full-thickness cartilage wear (thin arrows), subchondral sclerosis (arrowhead), and marginal osteophyte formation (double-tailed arrow). Taken from Loeser et al 2012. (Loeser, Goldring et al. 2012)

1.2.3 Clinical Features and Pain in OA

As informative as the above macro and microscopic transformations in joint structure may be to the study of OA, these are not the motives which drive patients to seek medical advice nor their primary concern while receiving treatment. They are similarly not the primary diagnostic considerations. Clinically we are concerned with symptomatic, not simply radiographic, osteoarthritis, which is identified by the presentation of:

- **Joint pain and tenderness.**
- **Limitations to movement** and/or joint instability.

- **Crepitus** - a grating, crackling or popping sound or sensation produced by friction between bone and cartilage or the fractured parts of a bone.
- **Effusions** – the build up of intra-articular fluid.
- **Morning stiffness**, lasting less than 30minutes.
- **Some low level inflammation**, in the absence of a raised white blood cell count.

Of these symptoms, pain is of greatest significance to patients. The quality and duration of painful episodes can differ greatly from patient to patient: depending on how advanced the disease, which joint structures are affected and patient-specific psychological vulnerabilities that may predict a greater pain experience in certain individuals.

The pain reported is largely movement-evoked, where patients show increased sensitivity to load bearing and movement in the normal range of the joint, a form of allodynia. Individuals also experience heightened sensitivity to particularly strenuous movements or noxious stimulations, described as mechanical hyperalgesia(Schaible, Richter et al. 2009). In other words, there is a leftward shift of the mechanical stimulus-response relationship, where the threshold at which pain is evoked is reduced and the magnitude of the response to suprathreshold stimuli increases. Although largely resolved upon rest, in small subsets of patients there is also ongoing pain, largely indicative of a more advanced pathophysiology(Hunter, McDougall et al. 2008). There are also those who suffer from nighttime based resting pain(Scott 2006).

The descriptors commonly associated with OA pain include aching, burning, throbbing, sharp or shooting pains, where it is worth considering the impact this ongoing pain has on the quality of life of patients(Hawker 2009). Notably, beyond the obvious discomfort, pain is detrimental to mood, quality of sleep, appetite and concentration of these individuals, demonstrated by the ability of effective analgesics to improve each of these co-morbidities(Hawker, Stewart et al. 2008, Schein, Kosinski et al. 2008).

Many individuals also experience referred pain, the experience of pain in neighbouring, unaffected areas of the body. Thus patients with OA in their hip, who classically experience pain in the groin region, may additionally experience pain referred to their knees and buttocks(Kean, Kean et al. 2004). This referred pain can be so great that there have been known cases of individuals reporting severe knee pain with no knee pathology but significant osteoarthritis of the hip(Kean, Kean et al. 2004). It is suggested this referred pain may be the effect of either convergent spinal inputs or the

result of a central hypersensitivity indicative of more wide spread CNS changes (**See Section 1.5.4.4**).

Much like the radiographic changes, these symptoms are commonly assessed and rated using a scale or index that allows for a quantitative assessment of severity, as well as efficacy and outcomes of treatment avenues – not least, physiotherapy, joint replacement and analgesia. The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) is perhaps the most commonly used of these scales (Bellamy 2008). This survey is completed by patients, either in person or over the phone, in approximately 10mins and assesses pain, stiffness and physical function. The index is composed of 24 sections divided into three subclasses:

- **Pain (5 items):** during walking, using stairs, in bed, sitting or lying, and standing.
- **Stiffness (2 items):** after first walking and later on in the day.
- **Physical Function (17 items):** including stair use, rising from sitting, shopping, putting on / taking off socks, rising from bed and light household duties.

Patients answer these questions, either using a 5 point Likert-type or visual analogue format, to produce a standardized assessment of self-reported severity, which crucially relates this to activities relevant to the patient. The relative success of WOMAC as a measure of symptomatic OA are demonstrated by the success of the measure to predict physical function and pain scores 2 years after joint replacement (Fortin, Penrod et al. 2002, Lingard, Katz et al. 2004).

The significance of measures such as WOMAC, which focus on symptoms such as pain and mobility, is that they assess issues that directly affect the quality of life of patients. While there may be value in halting the progression of structural pathology in OA, the significance of these changes to patients will be limited if they do not translate to improvements in scores such as WOMAC. As previously mentioned, this is an entirely credible possibility, given the tenuous relationship between radiographic and symptomatic OA.

It has also been documented that OA patients use similar descriptors for their pain as neuropathy patients (Hawker, Stewart et al. 2008, Parsons, Ingram et al. 2011), such as burning, electric shock or numbness, where the use of questionnaires such as PainDETECT identify patient sub groups with scores that indicate a neuropathic-like pain (Gwilym, Keltner et al. 2009). However, it is worth considering that scales like this have been developed to identify neuropathic elements to patients' pain and thus may have an inherent bias, where neuropathy may not be the exclusive cause of such sensation.

70% of knee OA patients have been shown to exhibit somatosensory abnormalities(Wylde, Palmer et al. 2012). These include localized thermal and tactile hypoaesthesia and pressure hyperalgesia at the affected knee, as well as some tactile hypoaesthesia and pressure hyperalgesia in the pain free forearm. Similarly, it has been documented that OA patients exhibit loss of sensations, notably to proprioception and vibration sensitivity at cutaneous sites(Oliveria, Felson et al. 2005, Hirsch, Just et al. 2013, Zavan, Ferroni et al. 2013). Diagnosis of a neuropathic pain requires the “presence of negative or positive neurological signs concordant with the distribution of pain”(Danziger, Weil - Fugazza et al. 2001). These sensory losses represent negative signs, while changes to pressure pain thresholds in these patients represent positive signs(Arendt-Nielsen, Nie et al. 2010), supporting the idea that a small subset of patients may have a neuropathic-like pain condition.

1.2. 4 Risk Factors

Osteoarthritis can be viewed as the shared outcome of a range of genetic, metabolic and environmental triggers and risk factors, though the mechanism underlying the initiation and perpetuation of this condition are not yet fully understood. Broadly speaking, the following are considered primary risk factors in the development of OA:

1. Aging

- All studies indicate that the incidence of OA increase severely with age, across all joints(Arden and Nevitt 2006). While 1 in 5 adults aged 50-59 suffers from painful OA in one or both knees, by the age of 80+ this incidence increases to 1 in 2 adults(Arthritis Research Nov 2008).
- This relationship is likely the result of a convergence of age related factors, not least declining muscle function(Hurley 1999), decreased joint stability(Sharma 1999, Sharma, Lou et al. 1999), increased vulnerability of joint tissues to biomechanical insults and shift in the equilibrium between catabolic and anabolic processes in the cartilage, altering the ultimate cartilage composition and thickness(Arden and Nevitt 2006).

2. Bone Density and Osteoporosis

- Bone changes are a crucial part of OA pathophysiology (subchondral bone sclerosis and osteophytes) and thus the health of the bones play a crucial role. It has been shown that greater bone mineral density predisposes women to OA, while osteoporosis protects against – though both of these may relate to Oestrogen(Hannan, Anderson et al. 1993, Dequeker, Boonen et al. 1996).

3. Gender

- Women are more prone to suffering from OA, most especially of the knee, hands or generalized OA (affecting multiple joints). Similarly, hip OA has been shown to progress more rapidly in women(Dougados, Gueguen et al. 1996).
- It is suggested that these gender differences relate to various levels of the sex hormones, given HRT can reduce the risk of OA(Arden and Nevitt 2006).

4. Genetics

- The combination of twin studies, epidemiology of family history and exploration of rare genetic diseases have assessed the heritability of OA as more than 50%, dependent upon the joint. Specifically, studies have shown heritability of Knee OA between 39 and 65%. (Spector and MacGregor 2004)
- Candidate gene studies and genome wide linkage studies have suggested a wide number of possible genetic links – see Valdes and Spector 2010 for a review of these(Valdes and Spector 2010). Similarly, our understanding of the genetics of OA pain is growing – see Thakur et al 2013 for a review(Thakur, Dawes et al. 2013).

5. Obesity

- Obesity is the major risk factor associated with OA(Blagojevic, Jinks et al. 2010), where weight loss is often advised to limit the progression of OA pathology. The effect of obesity appears to be greater in women and there is a tendency towards bilateral over unilateral OA.
- Recent work suggests that this is not simply a byproduct of joint loading, since there is a two fold greater risk of hand OA in obese vs. normal weight patients. It is proposed that there is a more complex relationship between OA, obesity and inflammation(Berenbaum, Eymard et al. 2013).

6. Race

- Discreet differences in risk exist by race and climate. For example, African American are less likely to develop symptomatic hand OA but more likely to suffer knee only OA vs. Caucasians(Nelson, Golightly et al. 2013). In regards to OA pain, it is suggested that the difference between Caucasian and African Americans may be the result of differences in Vit D

levels, specifically that Vit D deficiency may be a risk factor for greater OA Pain(Glover, Goodin et al. 2012).

- Evidence suggests that the prevalence of knee OA is lowest in South and Southeast Asia while hip OA is lowest in East Asia(Cross, Smith et al. 2014). However, this is expected to change rapidly over the next century as the proportion of the population in Asian countries becomes increasingly aged. For example, by 2040 Singapore will experience an 316% increase in the population over 65, and India a 274% increase(Fransen, Bridgett et al. 2011). This will likely combine with increasing prevalence of obesity in these populations, combined with the manual heavy occupations, to increase prevalence in Asia over time.

7. Smoking

- Smoking appears to have a moderately protective effect(Blagojevic, Jinks et al. 2010), though there is little to no biological explanation of this. It is possible that this is merely a correlate of the effect smoking has been shown to be linked to a lower BMI(Blagojevic, Jinks et al. 2010).

8. Trauma

- A history of joint injury or trauma is a central risk factor for the development of OA. 50% of those diagnosed with anterior cruciate ligament or meniscus tears have symptomatic OA within 10-20years of injury(Lohmander, Englund et al. 2007). Similarly, in another study, while only 6% of uninjured participants developed knee OA, 14% of those with history of knee injury in adolescence or young adulthood developed OA(Anderson, Chubinskaya et al. 2011).
- It is worth noting that while OA is more common in former athletes, largely as a consequence of intense joint loading and injury(Maffulli, Longo et al. 2011), this by no means outweighs the benefits of sport given the protective effects of exercise on patients already suffering OA(Fries, Bruce et al. 2012).

1.2.5 Epidemiology

Analysis of the Johnston County Osteoarthritis project, a longitudinal prospective study of OA in Johnston County, North Carolina, estimated that the lifetime risk of developing symptomatic knee OA to be 44.7% overall(Murphy, Schwartz et al. 2008). Although they reported no significant differences by gender, the lifetime risk for women was 7% points higher at 46.8%, while both obesity and history of injury significantly increased the lifetime risk.

At present, roughly 8.5million Britons have OA(Smith 2012). It is predicted that OA will become more prevalent as the population ages, given incidence increases sharply at >50years of age(Oliveria, Felson et al. 1995), and obesity becomes more prevalent. This is a pattern observed clearly in the USA, where the number of Americans with OA in either a hip, knee or hand joint increased from 21million in 1995 to 27million in 2005(Lawrence, Felson et al. 2008).

The Framingham Study is perhaps one of the best-known studies of prevalence and risk factors associated with OA. This population study, initially recruited as part of a prospective study of

Group (age)	Total (n)	% Radiographic OA	% Grade 3 & 4	% Symptomatic OA
All Subjects	1,420	33.0	15.7	9.5
63-69	530	27.4	11.5	7.0
70-79	684	34.1	17.8	11.0
≥80	206	43.7	19.4	11.2
Women	831	34.4	15.5	11.4
63-69	303	25.1	10.6	7.6
70-79	414	36.2	17.6	13.0
≥80	114	52.6	21.1	15.8
Men	589	30.9	16.0	6.8
63-69	227	30.4	12.8	6.2
70-79	270	30.7	18.2	7.8
≥80	92	32.6	17.4	5.4

Table 3.1: Prevalence of Knee OA by age and gender in the Framingham OA study – data from Felson 1990(Felson 1990). Prevalence of radiographic and symptomatic Knee OA increases with age, with greater prevalence in women

cardiovascular risk and incidents, monitored over 5,200 adults over a 35year period, where of the 1,805 subjects who participated in examinations, radiographs were obtained from 1,424 (4 of which were excluded due to bilateral knee replacements) and graded using the Kellgren Lawrence scale(Felson 1990). Patients were also asked a series of questions about knee symptoms and physical, functional disability. This allowed a comparison of the prevalence of radiographic or symptomatic knee OA, by age and sex – the results of which are given in the Table 1, below(Felson 1990).

This data clearly demonstrated both the increasing prevalence of symptomatic and radiographic knee OA with age, but also the increased prevalence in women. It also demonstrates that while roughly one third will exhibit radiographic OA, only around one tenth – rising to 16% in women over

the age of 80 – suffer symptomatic OA, which involves pain, aching, stiffness and functional impairment in addition to radiographic changes.

Ethnic differences in the prevalence of OA have also been noted, as briefly discussed in risk factors. According to Nelson 2013, African American are less likely to develop symptomatic hand OA but more likely to suffer knee only OA vs. Caucasians(Nelson, Golightly et al. 2013). This is also shown by the Johnston County OA project, which observed 32.2% vs. 23.8% prevalence of radiographic hip OA in African American men vs. Caucasian men(Jordan, Helmick et al. 2009). The Beijing Osteoarthritis study meanwhile has shown that both hand and hip OA are less prevalent in Chinese populations vs. white America, where the prevalence of hip OA in Chinese ages 60–89 years was 0.9% in women and 1.1% in men, over 80% less frequent(Nevitt, Xu et al. 2002, Zhang, Xu et al. 2003). Conversely, the Beijing study revealed a higher prevalence of both radiographic and symptomatic knee OA in women compared to their white Framlington counterparts, while no difference was seen in male groups(Zhang, Xu et al. 2001).

1.3 Current Treatments of OA Pain

The primary complaint of OA patients is pain. As previously described, 71% of OA patients are in almost constant pain, where 1million rate their pain as unbearable(Smith 2012). Whether in the early stages of OA, or the latter stages awaiting joint replacement, it is pain that drives patients to seek out their GPs, in need of analgesia. The problem facing clinicians, however, is that 2 in 5 say the analgesia they are provided is either not very effective or not at all effective(Smith 2012). Similarly, because the majority of patients are among the more elderly, they are more likely to exhibit concurrent conditions, such as CVD, which will complicate and limit the prescriptions the GP can offer. Nevertheless, there are a number of interventions open to patients, ranging from non-pharmacological, pharmacological to surgical. I will be focusing on pharmacology and surgery, where the non-pharmacological interventions are outlined in **Figure 1.4** and **1.5**.

1.3.1 Pharmacology

Paracetamol is the first choice of oral analgesia for patients, whether recommended or bought over the counter to ease pain in the early days of OA. For a long period, paracetamol (up to 4g per day) was considered the analgesic of choice in the mild to medium stages of OA, not least because of its safety and effectiveness(Jordan, Arden et al. 2003, Zhang, Doherty et al. 2005, Zhang, Moskowitz et al. 2008). Most literature reviews pointed to a slightly lesser analgesia, but greater safety profile of paracetamol vs. NSAIDs, thus recommending it as the long-term analgesic of choice(Zhang, Jones et al. 2004, Zhang, Doherty et al. 2005). Specifically, one meta-analysis of 10 randomized controlled trials (RCTs) suggested that the safer profile of paracetamol, despite being less effective, recommended it for first line treatment above NSAIDs(Zhang, Nuki et al. 2010).

However, findings in more recent literature are beginning to question the consensus. A recent GDC review (The NICE Guideline Development Group) has pointed to a smaller efficacy of paracetamol analgesia in OA than was previously believed and consequently NICE will be conducting a review in the near future of the evidence on pharmacological management of osteoarthritis(Excellence 2014, Excellence 2014). Further, though the side effect profile is smaller than that of NSAIDs, patients are still at increased risk of upper GI complications, notably bleeds(Rahme, Barkun et al. 2008), while some patients may exhibit mild loss of liver function or hepatotoxicity(Zhang, Nuki et al. 2010).

NSAIDs – Non Steroidal Anti-Inflammatory Drugs – are prescribed in two distinct forms, topical and oral. Topical NSAIDs, while effective on their own in mild OA, can make successful adjuncts to paracetamol. The peripheral application has the notable benefit of avoiding cardiovascular and gastrointestinal (GI) risks, whose impact are far greater in the largely older patient pool(Baraf, Gloth

et al. 2011). As such, it is only after paracetamol and topical NSAIDs have been insufficient that use of oral NSAIDs and COX-2 inhibitors are recommended by NICE(Excellence 2014).

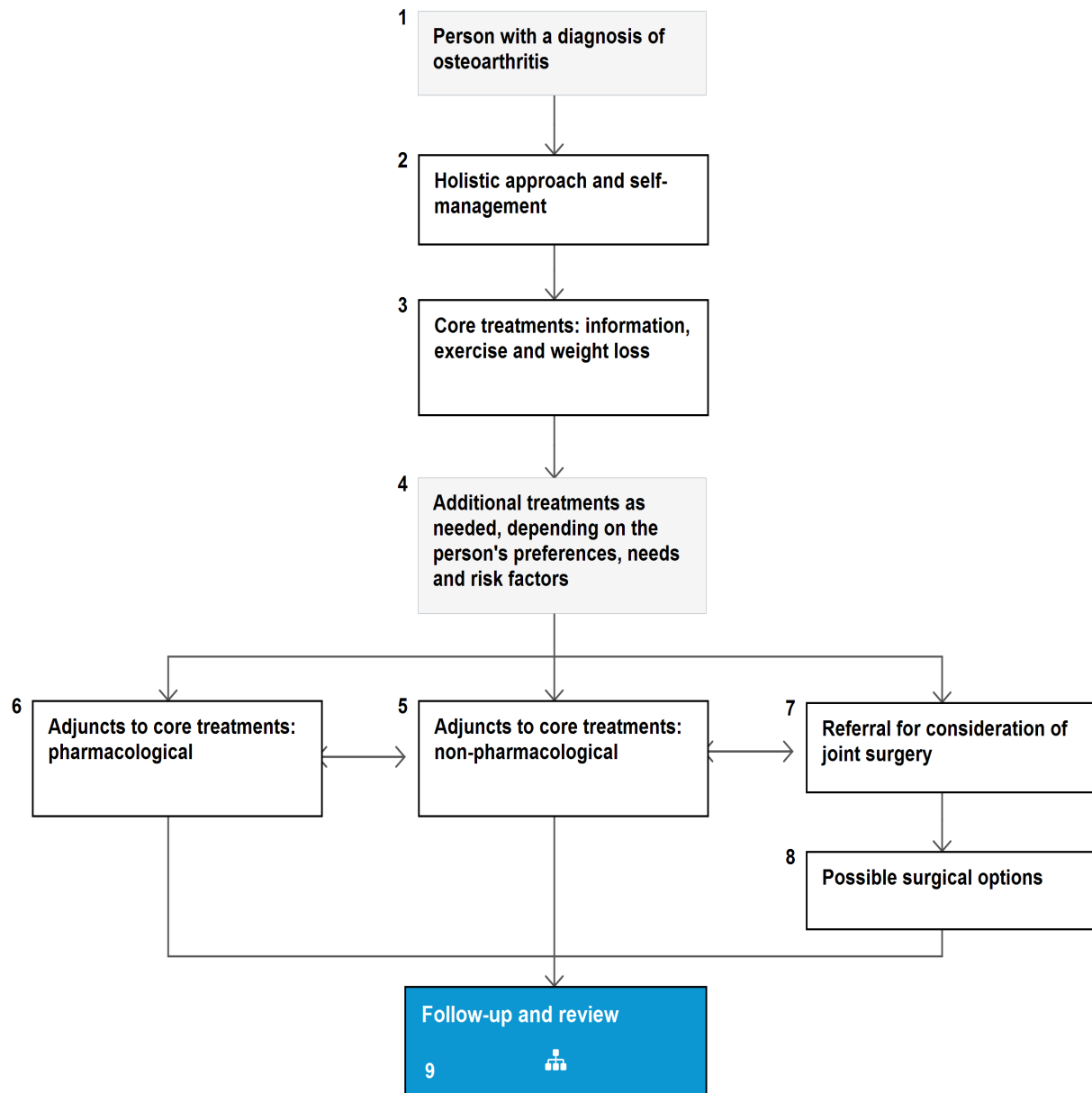


Figure 1.3: National Institute for Health and Care Excellence guidelines for the management of Osteoarthritis (NICE 2014). Following a diagnosis of OA (1) the clinician must assess the impact of OA on the patient's function, quality of life, mood, occupation and relationships (2). Self-management interventions are then suggested, such as weight loss, exercise and suitable foot wear, in addition to treatments in line with the severity of the patients OA. These will include prescription of pharmacological interventions, such as paracetamol, NSAIDs, COX-2 inhibitors and opioids (6), non-pharmacological interventions such as

thermotherapy, TENS and assistive devices (5), or in more advanced cases, referral for joint replacement or arthroplasty.

NSAIDs have been found to be effective analgesics in OA across a range of studies, both in patients and animals(Zhang, Jones et al. 2004, Towheed, Maxwell et al. 2006, Gallelli, Galasso et al. 2013). However, the prevalence of serious GI effects renders NSAIDs less tolerable. A Cochrane review of fifteen RCTs involving 5986 participants found 19% of patients experienced an adverse GI event, vs. 13% in the paracetamol group(Towheed, Maxwell et al. 2006). As such, recommendations focus on the use of NSAIDs at the lowest possible dose for the shortest period of time, and the co-prescription of a proton pump inhibitor to protect the GI tract(Bijlsma, Berenbaum et al. 2011, Excellence 2014).

The Coxibs, selective Cox-2 inhibitors, can provide some respite from the gastric risks of conventional NSAIDs with the same or greater analgesic efficacy(Sarzi-Puttini, Cimmino et al. 2005). Randomized controlled studies have shown that rofecoxib and celecoxib produce 2 to 3 times fewer GI events and complications, such as perforations and bleeds, than ibuprofen or naproxen(Jones, Rubin et al. 2008). Similarly, Laine et al's review of RCTs, meta-analyses, and literature review demonstrated a 74% reduction in the relative risk of gastroduodenal ulcers, and 61% reduction in ulcer complication using the coxib versus traditional NSAIDs(Laine, White et al. 2008). However, contrary to this, their study also demonstrated a twofold increase in the risk of myocardial infarctions vs. placebo(Laine, White et al. 2008), a consideration with added significance given the average age and weight of an OA patient.

In addition to the use of oral analgesics, patients may also be prescribed **Capsaicin** creams (0.025%, 4x daily). Capsaicin is the lipophilic alkaloid agent extracted from chilli peppers, responsible for the hot or "spicy" sensation of this plant. Capsaicin binds to transient receptor potential vanilloid type1 (TRPV1) ion channels to cause the activation and sensitization of small diameter afferents, termed C fibers, to produce a burning pain. However, the long term activation of TRPV1 can cause the desensitization of these fibers, and eventually the "drawing back" of the afferent terminals from the site of application(Nolano, Simone et al. 1999), producing loss of sensation and as such is considered an adjunct therapy in 8/9 treatment guidelines, often alongside paracetamol(Zhang, Moskowitz et al. 2007). While several studies show statistical significance in the efficacy of capsaicin cream treatment(McCarthy and McCarty 1992, Altman, Aven et al. 1994, Schnitzer, Morton et al. 1994, Mason, Moore et al. 2004), it is worth keeping in mind the inability to blind participants in capsaicin studies, which may limit these studies.

For those patients prone to inflammatory flares, **intra articular injections of corticosteroids** can supplement paracetamol/NSAIDs to provide long lasting (4-6week) anti-inflammatory action and analgesia, with an effect size of 0.58(Bijlsma, Berenbaum et al. 2011), for those patients experiencing

moderate to severe pain(Excellence 2014). A Cochrane review of 28 trials of 1973 participants, comparing intra articular corticosteroid to placebo, found these injections to be effective analgesics in weeks 1-3, with a numbers needed to treat of just 3-4(Bellamy, Campbell et al. 2009). However, they found no significant effect beyond week 4. IA corticosteroid may represent a short term, well tolerated analgesic intervention for patients, given the small side effect profile – as you would expect from a peripherally administered therapy. However, given the short term nature of this therapy, for what is a chronic pain condition, along side the relatively invasive administration vs. oral or topical analgesia, IA corticosteroids are rarer, and only recommended as an adjunct to the core therapeutic pathway(Excellence 2014).

For those patients experiencing moderate to severe pain, likely while waiting for surgical intervention, **opioid** therapies may similarly be considered where paracetamol alone failed. Opioids are known as the “gold standard” analgesic across a range of chronic pain conditions. These compounds provide a high standard of pain relief for patients with moderate to severe pain conditions, providing analgesia where many other compounds fail. This is the case in OA, where opioids are recommended in patients where the conventional first choice therapies have failed(Avouac, Gossec et al. 2007).

At the milder end of the Opioid spectrum, **codeine** or paracetamol-codeine combinations provides a superior level of analgesia than paracetamol for those patients for whom paracetamol alone or NSAIDs are insufficient(Bijlsma, Berenbaum et al. 2011). However, some studies have questioned whether these weaker opioids are truly more effective than paracetamol or NSAIDs, where one meta analysis of opioids for chronic, non-cancer pain demonstrated that only the strong opioids were more effective(Center 2008).

In more exceptional circumstances, patients may be prescribed stronger opioids like **tramadol**, **oxycodone** or **morphine** for short periods, which have been shown to be effective across a range of RCTs (Thorne, Beaulieu et al. 2008, Zhang, Moskowitz et al. 2008, Bijlsma, Berenbaum et al. 2011). Analysis of 18 placebo-controlled RCTs, including 3244 patients with OA, showed a moderate effect size of 0.78 for reduction in pain intensity(Zhang, Moskowitz et al. 2008). However, the tolerability of this class of drugs is extremely poor. Studies report withdrawal rates exceeding 30% as a result of nausea, vomiting, dizziness, constipation and somnolence(Zhang, Moskowitz et al. 2008, Bijlsma, Berenbaum et al. 2011), while others highlight that the gains in function through analgesia may be countered by the functional impairments of the side effects(Avouac, Gossec et al. 2007). Crucially, the numbers needed to harm – the number of patients you must treat before one will experience an adverse effect and withdraw – can be as little as 4 for strong opioids(Avouac, Gossec et al. 2007).

Furthermore, the desirability of opioid therapy is limited for long-term use by strong, not unfounded concerns around tolerance and addiction.

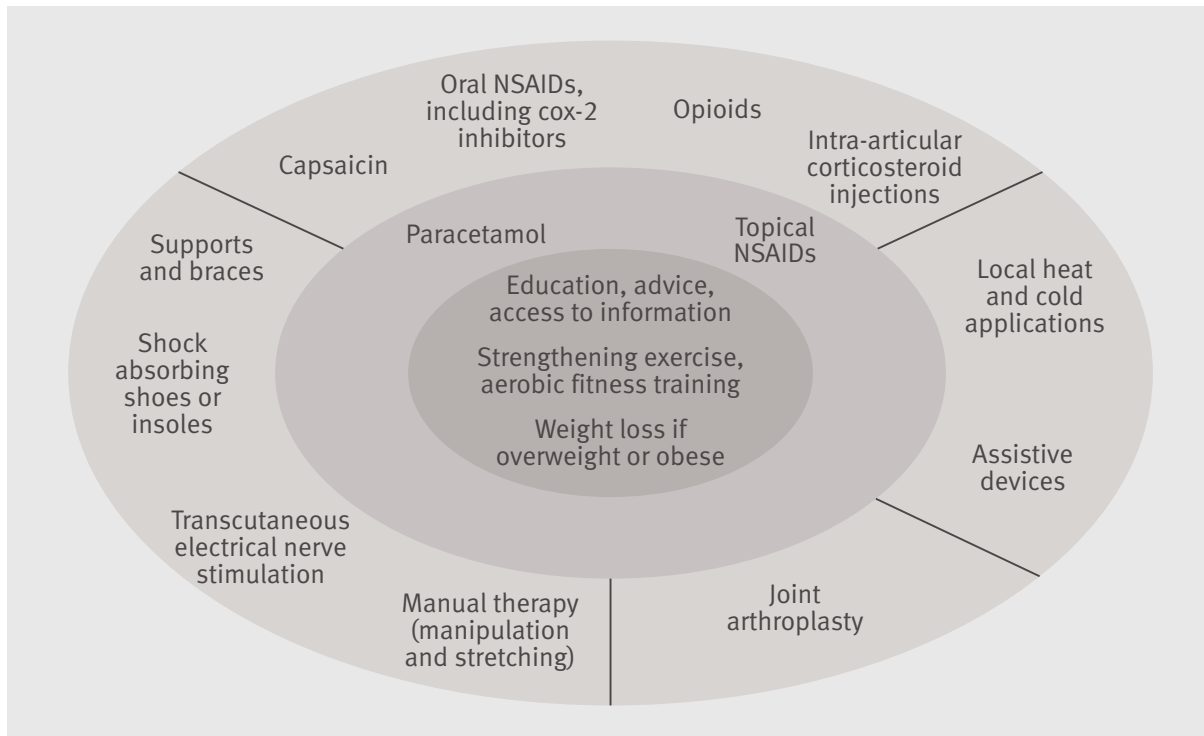


Figure 1.4: NICE Guidelines for the treatment of OA in adults(Conaghan, Dickson et al. 2008). Treatments are arranged in order of consideration, starting in the center, taking into account individual needs and risk factors. Along side the central “core” treatments, the next major consideration is paracetamol and/or topical NSAID. The outer circle also shows adjunctive treatments (both non-pharmacological and surgical), which have less established efficacy, provide less symptom relief, or increased risk to the patient compared with those in the second circle.

A newer avenue of consideration for the pharmacological management of OA is the use of **antidepressants**. These drugs, which are already licensed for management of neuropathic pain conditions such as diabetic neuropathy and fibromyalgia, manipulate the monoamine transmitter systems to produce analgesia and, at higher doses, combat depression and anxiety. One particular drug, **duloxetine**, has received particular attention following on from success in RCTs(Chappell, Ossanna et al. 2009, Sullivan, Bentley et al. 2009, Hochberg, Wohlreich et al. 2012, Pergolizzi, Raffa et al. 2013). Duloxetine is a selective serotonin noradrenaline reuptake inhibitor (SNRI) that, by blocking reuptake of these monoamines, increases availability of these transmitters at the post synaptic receptors. These RCTs have shown duloxetine to provide significant reduction in pain scores, starting immediately and maintaining across the entirety of the studies durations, with

concomitant improvements in function and WOMAC scores(Chappell, Ossanna et al. 2009, Sullivan, Bentley et al. 2009, Hochberg, Wohlreich et al. 2012). Of notable advantage is the ability of this compound to ease affective comorbidities of chronic pain, which contributes further to the improvement in quality of life. Crucially, these drugs seem far more tolerable, with a NNH of 8(Hochberg, Wohlreich et al. 2012), and while 50.2% of OA patients on duloxetine vs. 36.7% on placebo reported treatment emergent adverse events there is a long term decline in their incidence – for example nausea occurs in 31.8% in the first 8 weeks but in just 3.4% from the 8th week till the end of the year(Pergolizzi, Raffa et al. 2013). Consequently, duloxetine is now licensed for the treatment on OA pain in the USA, though NICE does not currently endorse the use of antidepressants such as duloxetine in the OA treatment pathway.

1.3.2 Surgical Intervention

Joint replacement surgery is the most common elective surgical procedure performed on the NHS, costing over £1billion for the approx. 160,000 procedures performed in the UK per year[6-8]. This is the removal of the articular surfaces to be replaced by synthetic materials – notably metal, plastic and ceramic. It is universally accepted as the end point of the treatment pathway, when pharmacological and non pharmacological interventions have failed to provide adequate pain relief and improvement in function(Zhang, Moskowitz et al. 2008, Excellence 2014). Not only do all studies report significant improvements in pain and physical function scores, joint replacements are more cost effective than current pharmacological therapies over the long term – where Rasanen et al suggest that the cost per quality adjusted life year works out as €13,995 for total knee arthroplasty(Rasanen P 2007).

However, in a cruel twist of fate, these surgical procedures designed to alleviate pain can often achieve the exact opposite(Wylde, Hewlett et al. 2011, Beswick, Wylde et al. 2012). In a study of 632 total knee replacement (TKR) patients and 662 total hip replacements (THR), Wylde et al found 44% of TKR and 27% of THR patients experienced some form of persistent post surgical pain, while 14 and 8% respectively suffered severe-extreme persistent pain(Wylde, Hewlett et al. 2011). Crucially, those patients with other pain and health problems, pain vulnerability/sensitivity and depression or poor mental health scores appear to be at high risk of developing this(Zhang, Moskowitz et al. 2008, Wylde, Hewlett et al. 2011). Similarly this is a highly invasive procedure, performed under general anaesthetic. Given the average age and weight of OA patients reaching this point in the treatment pathway, this adds extra risk to the procedure.

1.4 Modeling Osteoarthritis Pain

In order to better understand the mechanisms underlying the generation, maintenance and pharmacological modulation of pain in OA, there is a requirement to model the disease in animals. Researchers have generated a number of models that reliably produce OA-like pain in animals, but these models vary considerably – both in the means of induction, the symptom profile and the pharmacological sensitivities. These include surgical models, such as the anterior cruciate ligament transection model(Pond and Nuki 1973), which work on the principal of destabilization of the joint; Spontaneous arthritis, such as in aged Dunkin-Hartley guinea pigs(Jimenez, Glasson et al. 1997), which develop arthritis naturally in relation to their gain in body mass; Transgenic mice, such as MMP-13 over-expressing mice(Neuhold, Killar et al. 2001), which have a metabolic propensity to OA; Enzymatically induced models, such as intra-articular injection of collagenases(Peter, der Kraan et al. 1990); and chemically induced models, such as the monosodium iodoacetate (MIA) model of OA(Kalbhen 1987).

1.4.1 The Monosodium Iodoacetate Model

The model utilized throughout this thesis is the **Monosodium Iodoacetate (MIA)** model of OA, first characterized in chickens in 1977 by Kahlben and Blum and finally used in rats in 1987(Kalbhen 1987). This model involves the injection of MIA into the intra articular space of an anaesthetized animal, where the iodoacetate acts to inhibit glycolysis by the competitive inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glycolysis, the key first stage of respiration, enables cells to produce ATP in the absence of oxygen. Due to the A-vascular nature of cartilage, chondrocytes rely heavily on diffusion to receive both glucose and oxygen, leaving them reliant almost exclusively on glycolysis to respire. As such MIA limits, if not halts entirely, the production of ATP in chondrocytes in the joint to cause their eventual death, followed by the gradual loss of proteoglycans, fibrillation and thinning of the cartilage, along side osteophyte formation and thickening of the subchondral bone(Dunham, Hoedt-Schmidt et al. 1992). This model is now considered to be the most common for modeling OA in animals(Little and Zaki 2012).

The notable advantages provided by the MIA model are several fold; The model induces rapidly, with pain behaviour, swelling and chondrocyte degeneration from the first day, and bone lesioning by day 14; The ease of induction, given the simple and relatively trauma free procedure – where the rat is under anesthesia less than 5mins; The model produces consistent hypersensitivity, both at the joint and ipsilateral paw, across numerous labs, where it is notable that various doses used can be selected to modify the progression and severity of the pain condition(Guingamp, Gegout-Pottie et al. 1997,

Janusz, Hookfin et al. 2001, Schuelert and McDougall 2009); But the crucial feature of the MIA model is its generation of both joint histopathology and pain characteristics that are not dissimilar from those observed in OA patients(Guingamp, Gegout-Pottie et al. 1997, Mohan, Perilli et al. 2011).

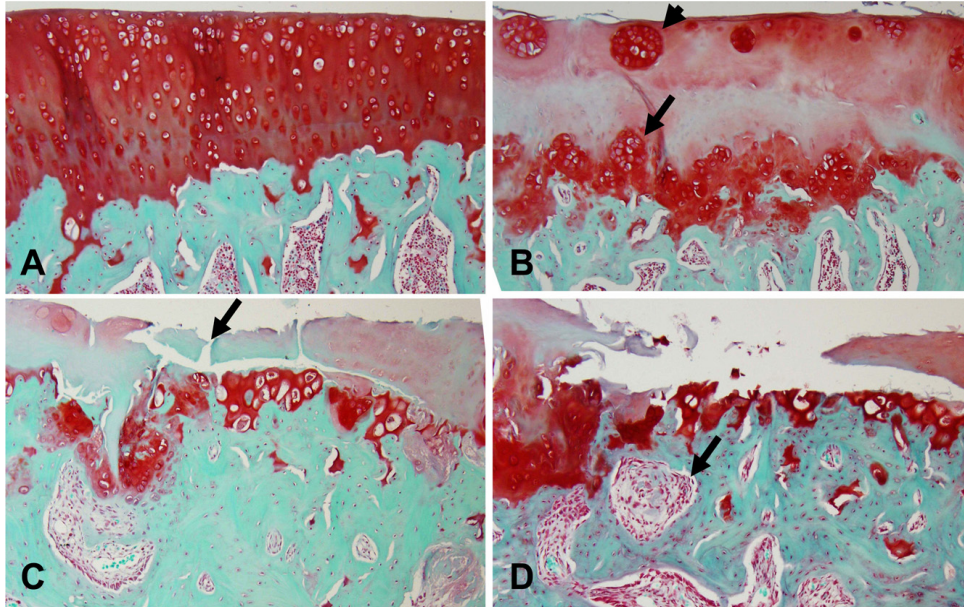


Figure 1.5: Coronal sections of control (A) and 0.2mg injected MIA tibia (B-D) at 10weeks post injection, stained with Safranin O and fast green. A) The control tibia exhibits healthy cartilage, with normally distributed chondrocytes. MIA injected tibia shows loss of proteoglycan, viable chondrocytes and irregular clusters (B) of chondrocyte proliferation, fibrillation, vertical fissures and bone sclerosis (C), delamination and the replacement of cellular bone marrow by spindle cells in fibrous stroma (D). Taken from Mohan et al 2011(Mohan, Perilli et al. 2011).

1.4.2 Histopathology in the MIA model

Within 24hrs of the injection of MIA, changes are occurring within the joint. The process begins with the reduction of proteoglycan staining as glycolysis inhibition limits the production of these extracellular matrix components(Guingamp, Gegout-Pottie et al. 1997, Janusz, Hookfin et al. 2001). This is rapidly followed by observable necrobiosis of chondrocytes over the proceeding 1-5days, in tandem with the gradual thinning of cartilage as the extracellular matrix loses its integrity(Dunham, Hoedt-Schmidt et al. 1992, Janusz, Hookfin et al. 2001, Guzman, Evans et al. 2003). Though observed across the joint, the effects of proteoglycan and cartilage loss are most marked in the central medial tibial plateau, while cartilage degeneration is milder on the lateral side(Mohan, Perilli et al. 2011).

During this time there is evidence of marked inflammation. This is observed at a macroscopic level as the thickening of the synovium and patellar fat pad, and swelling of the injected knee, while at the microscopic level there is observable infiltration of monocytes and neutrophils as part of a

proteinaceous and fibrin rich oedema(Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Clements, Ball et al. 2009). However it is worth noting that across these studies, this inflammation is largely resolved by day 7, peaking during days 1-3(Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Clements, Ball et al. 2009). Consistent with this inflammation, TNF α IL-6 and NGF are upregulated in the joint(Orita, Ishikawa et al. 2011), Substance P expression is enhanced in the synovium(Ahmed, Li et al. 2012) and MIA animals exhibit marked hypersensitivity to ACh, BK and 5HT(Okamoto and Atsuta 2010).

By day 7, the point at which we are witnessing the resolution of inflammation, we begin to see the progressive involvement of subchondral bone, with increasing numbers of osteoclasts along the junction of necrotic cartilage and the bone(Guzman, Evans et al. 2003). Progressively, the cartilage becomes fibrillated and chondrocytes calcify and large osteophytes form, with bone sclerosis and exposure by days 14-21(Dunham, Hoedt-Schmidt et al. 1992, Janusz, Hookfin et al. 2001). Over the longer term, there is also considerable remodeling of the bone structure, with increased trabecular thickness, reduced trabecular numbers and separation, cysts, and the replacement of marrow by spindles(Guzman, Evans et al. 2003, Mohan, Perilli et al. 2011).

1.4.3 Pain-like Behaviour in the MIA model

As this study's focus is on pain, it is critical that the MIA model produces a reliable and representative pain profile in animals, representative of the patient experience. MIA, at various doses, has been well characterized as inducing a biphasic shift in weight bearing, peaking in line with the inflammatory profile at day 3 and regaining from days 10 onwards(Bove 2003, Pomonis, Boulet et al. 2005, Kelly, Dunham et al. 2012, Thakur, Rahman et al. 2012). Alongside observed adjustments in gait, as analysed by CatWalk in MIA animals(Ferreira - Gomes, Adaes et al. 2010, Ferland, Lavery et al. 2011), this shift in weight borne to the contralateral limbs is indicative of joint hypersensitivity, where both animals and patients minimize joint usage to shield themselves from the pain associated with knee pathology(Zeni and Higginson 2011). In addition, MIA animals also exhibit depressed behaviours such as wheel running and nocturnal exploration(Guingamp, Gegout-Pottier et al. 1997, Stevenson, Mercer et al. 2011), consistent with the clinic experience that pain depresses the activity e.g. chores, shopping in patients(Yelin, Lubeck et al. 1987). These too show a bilateral pain profile, in which rats exhibited an initial peak in loss of nocturnal mobility in the first 3 days following injection, a period of recovery, and an increasing loss of activity following Day 11(Guingamp, Gegout-Pottier et al. 1997).

Beyond the knee itself, there is also a well-characterized secondary hypersensitivity that develops in areas distal to the knee, such as the hind paw and bicep of MIA animals. These include both

mechanical hyperalgesia, as assessed by the Randall Selito apparatus, and tactile hypersensitivities, as assessed by von Frey(Combe, Bramwell et al. 2004, Fernihough, Gentry et al. 2004, Rahman, Bauer et al. 2009, Vonsy, Ghandehari et al. 2009, Thakur, Rahman et al. 2012, Kelly, Dobson et al. 2013). Nociceptive reflexes serving the biceps femoris and tibialis anterior are similarly sensitized(Kelly, Dobson et al. 2013), though these may be masked by descending controls. As with shifts in weight bearing and nocturnal activity, some studies have suggested that this secondary hypersensitivity exhibits an initial peak around days 2-3 to coincide with the models initial inflammatory flare; a baseline hypersensitivity profile, which may slowly build, is then established beyond ~day 10 onwards once joint pathology becomes more established(Vonsy, Ghandehari et al. 2009, Burnham 2012). As previously discussed (See 1.2.3), referred pain – the pain in a secondary area such as the paw – is a common feature in patients(Kean, Kean et al. 2004, Gwilym, Keltner et al. 2009).

1.4.4 Electrophysiology in the MIA model

Electrophysiology in the MIA model has taken two main streams, the investigation of changes in activity and evoked responses of peripheral fibers serving the joint; and recordings of spinal neurons, serving either the joint or areas of secondary hypersensitivity.

Single unit recordings of knee joint primary afferents have established a dose dependent increase in the firing of these afferents in response to both non-noxious and noxious torque in MIA animals(Schuelert and McDougall 2006, Schuelert and McDougall 2009). Similarly, mechanically sensitive A fibers have been shown to exhibit marked decreases in their mechanical threshold and increased response to suprathreshold stimuli(Kelly, Dunham et al. 2012). These studies have thus contributed to our understanding of peripheral sensitization in the MIA model but also suggests this peripheral sensitivity is proportional to pathology, given the links to dose. Interestingly, Kelly et al 2012 also demonstrated that while mechanosensitive C fibers did not exhibit reduced thresholds or increased responses, they did exhibit an increased occurrence and rate of spontaneous firing in MIA animals vs. naïve that was not observed in A fibers and which correlated to the changes in weight bearing behaviour(Kelly, Dunham et al. 2012). Clinically, this may mean C fibers are responsible for pain at rest, driving central sensitization, while A fibers are responsible for movement evoked pain.

Recordings of secondary neurons in the dorsal horn, which project to the brain, also indicate modifications of spontaneous and evoked firing. Studies utilizing single unit recordings of WDRs serving the ipsilateral hind paw have largely agreed upon the enhanced evoked responses of these neurons to graded punctate stimuli in MIA vs. shams, including at both the early and late stages of the model(Harvey and Dickenson 2009, Rahman, Bauer et al. 2009, Sagar, Staniaszek et al. 2010, Burnham

2012). There was similarly enhanced WDR spontaneous firing(Chu, Chandran et al. 2011). These findings are in agreement with the observed changes in mechanical sensitivity in behavioural tests, together indicating the presence of a central sensitization in MIA animals. It is highly likely that the afore mentioned enhancements in peripheral neuronal activity drive this central sensitization but that the overall pain profile observed in MIA is the collective result of both peripheral and central plasticity.

However, there is no clear-cut consensus on the effect of MIA on responses of deep dorsal horn neurons. While much of the work in this lab has demonstrated potentiated mechanical responses across a range of punctate stimuli, one thesis in this lab saw no change in either Lamina V WDR or Lamina 1 NS cells, only observing a significant change from sham at 8g vF in the Lamina 1 WDRs(Thakur 2012). The lack of increased baselines was similarly observed in Patel 2012 in her thesis evaluating the 2mg MIA model, in which she had similarly demonstrated enhanced baselines for cancer induced bone pain(Patel 2012).

There is similar divergence in the literature on whether responses are enhanced to thermal stimuli, for both behaviour and electrophysiology. Studies using high dose MIA (4.8mg), which from the work of Thakur et al 2012 can be inferred to be a neuropathic model of OA(Thakur, Rahman et al. 2012), have previously demonstrated decreased paw withdrawal latencies to noxious thermal stimuli(*A. OKUN1 2011, Liu, Okun et al. 2011). This is in agreement with those electrophysiology studies by Rahman et al and Burnham et al using a 2mg dose that show enhanced thermally evoked WDR responses(Rahman, Bauer et al. 2009, Burnham 2012). However, in direct contrast, in other studies, neither behaviour nor electrophysiology identified thermal hypersensitivity in the paw in other studies using ≤ 2 mg MIA (Gwilym, Keltner et al. 2009, Harvey and Dickenson 2009, Thakur 2012). This lack of thermal hypersensitivity appears to agree with clinical observations, where Gwilym et al 2009 found no significant difference in the reported thermal pain or temperature detection thresholds(Gwilym, Keltner et al. 2009), even though the patients assessed reported referred pain – signifying an established central sensitization. Similarly, QST characterized only 7% of patients with thermal hyperalgesia but 31% with thermal hypoaesthesia(Hochman, Davis et al. 2013).

These differences in observations in animal work are hard to explain. The fact that thermal (hot) hypersensitive behaviour is only exhibited in the high dose, 4.8mg model may suggest this is a dose related symptom. However, the differing electrophysiology profiles of MIA animals discussed all come from trials using a 2mg model so differences cannot be attributed to dose. It is possible this is the result of inter-experimenter effects, given differences are also observed in the baseline profiles of naïve and sham animals too, where the response profile of cells selected by different experimenters may lead to these observed differences in evoked responses.

In addition to these evoked changes, spinal electrophysiology has also elucidated a change in the receptive field size of spinal neurons serving the ipsilateral paw in MIA, but only in the NS and WDR cells of Lamina I (Not lamina V)(Thakur 2012). In other words, these secondary projecting neurons are responding to inputs from a wider array of primary afferents and thus a larger number of Lamina I neurons are activated by any given stimulus, indicative of central sensitization and a process which may underlie a degree of the hypersensitivity observed in MIA animals.

Though less well characterized, studies of WDR cells serving the knee have additionally revealed an increased spontaneous rate of firing(McGaraughty, Chu et al. 2010), though evoked responses to mechanical stimuli do not appear to differ from shams(McGaraughty, Chu et al. 2010, Chu, Chandran et al. 2011). However, it is possible that the increased peripheral inputs and central sensitization we would expect to see from these WDR recordings are masked by moderating descending controls which may provide analgesia to the primary site.

This thesis will engage both behavioural and electrophysiological techniques to profile the extent of hypersensitivity induced following the injection of a 1mg dose of MIA to the knee and the consequential development of an OA like condition. As such, it is important to understand that while the expected behavioural profile of this model is relatively uncontroversial in the published literature, there is no clear consensus in the literature on the expected evoked electrophysiological profile of WDR cells in these rats. Given this forms the baseline readings for the pharmacological investigation of descending control of pain in this model of OA, it will be particularly important to characterize clearly what difference, if any, is observed between the punctate and thermally evoked MIA and sham animals at the time points investigated.

1.5 An Overview of Nociception

When viewed simply, pain can be regarded as the consequence of a three major stages: The translation of tissue damage or noxious stimuli information by peripheral afferents; the modulation and transmission of information from peripheral afferents to second order neurons in the spinal cord; and the generation of a contextual, subjective pain experiences by the brain. These processes are consequence of complex layers of inputs and modulation, while the systems themselves are highly plastic, not least during a chronic pain condition such as OA.

1.5.1 Peripheral Nociception

Though the cartilage itself is not innervated, at least in healthy joints, the surrounding tissues, notably the synovium and bone, are well served by a diverse population of sensory afferents. Meanwhile, vascularization and neuronal infiltration provide sensation from the previously insensitive cartilage.

At the most basic level, OA is a nociceptive pain condition, where mechanoreceptors are directly activated by increased friction elicited upon movement, by bone compression, lesions, fractures and increased intra-osseous pressure. These high threshold mechanical stimuli activate primary sensory afferents projecting to the spinal cord.

1.5.1.1 The Fibers

Peripheral afferents convey sensory information to the CNS. They are most commonly sub-classified on the basis of their conduction velocity, which itself is a function of their distinct morphologies and myelination(Harper and Lawson 1985):

- **A β fibers:** Highly myelinated, wide diameter neurons which conduct at roughly 20-65ms⁻¹.
- **A δ fibers:** Medium diameter, myelinated neurons conducting in the range of 2.2- 8ms⁻¹.
- **C fibers:** Small diameter, un-myelinated fibers with the slowest conduction velocity, at less than 1.4ms⁻¹.

While it is possible to generalise the roles of these fibers it is noteworthy that even these sub classes exhibit considerable heterogeneity, most notably in terms of the thresholds to activation, stimulus specificity, transmitters and molecular markers.

1.5.1.2 Subdivision by Sensation

As part of an experiment to define whether intraepidermal electrical stimulation (IES) provides a fully selective nociceptive input, Mouraux *et al* defined some of the sensational subdivisions found between A β , A δ and C fibers(Mouraux, Iannetti et al. 2010). Namely, that activation of C fibers elicited prickling, tingling, warming and burning sensations, while selective A δ selective activation resulted in tingling, prickling and light touch. While such sensations relating to A β activation were expected, it is most notable that these fibers were also responsible for shock like sensations. The study highlights that while there is some subdivision of sensation at this superficial level, there is also considerable cross over – for example between prickling and tingling sensations between C and A δ fiber populations.

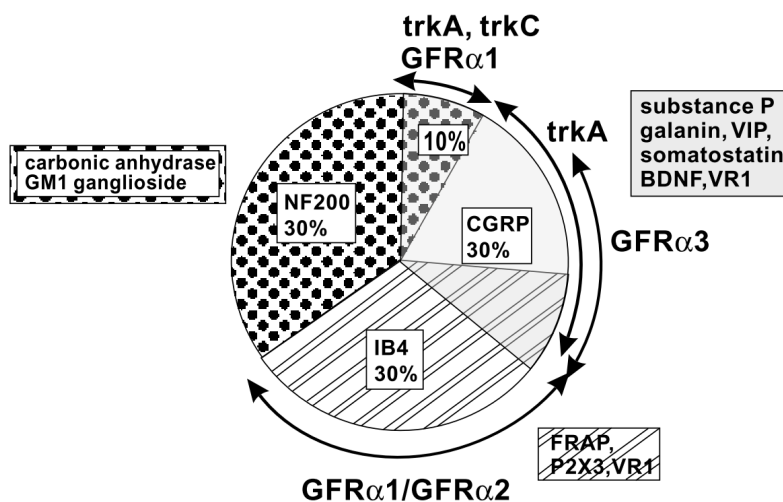


Figure 1.6 - Pie chart summarizing the subdivision of DRGs according to molecular markers, taken from Priestley et al 2002.

1.5.1.3 Molecular Markers Defining Subpopulations

Studies in cutaneous nociceptors proposed that at the simplest level this broad peripheral afferent population can be subdivided into two classes: those nociceptors that contain peptide transmitters (SP, CGRP) and those nociceptors that do not(Snider and McMahon 1998). These peptidergic fibers

concomitantly express TrkA receptors, denoting the importance of NGF for fiber survival(Snider and McMahon 1998), and are estimated to make up approximately half of all C fibers and about 20% of A δ fibers(McCarthy and Lawson 1989, Lawson 1996, Priestley, Michael et al. 2002). The remaining 80% of non peptidergic A δ receptors bind the plant lectin IB4(Silverman and Kruger 1988), and express FRAP(Silverman and Kruger 1988), GFR α 1-4 and Ret for glial cell line derived nerve growth factor(Priestley, Michael et al. 2002) – further emphasizing the difference in neurotropic reliance between these fiber subgroups.

However, there are a multitude of additional structural and neurochemical markers that denote potential subpopulations, some examples of which are summarized in the Table 1.2.

Molecular marker	Population	Reference
NF200	Large-medium myelinated fibers	Lawson and Waddell 1991
VGLUT1	Large-medium myelinated fibers	Brumovsky et al 2007
VGLUT2	Medium and small afferents	Brumovsky et al 2007; Lagerström et al 2011
VGLUT3	Non-peptidergic LTM C fibre subpopulation	Seal et al 2009
Tyrosine hydroxylase	Non peptidergic LTM C fibre subpopulation	Li et al 2011
CGRP, SP	Peptidergic nociceptors	Lawson 1997
Prostatic Acid Phosphatase	Non-peptidergic nociceptors	Zylka et al 2008
IB4 binding	Non-peptidergic nociceptors	Silverman and Kruger, 1988a
MrgprD	Non-peptidergic nociceptor population exclusively serving the epidermis	Zylka et al 2005
MrgprdB4	Non-peptidergic C fibre population mediating pleasant touch	Vrontou et al 2013
Nav1.8	Subset of sensory afferents - ~90% nociceptors, LTM C Fibers and RA A β LTM afferents	Shields et al 2012
FRAP	Non-peptidergic nociceptors	Silverman and Kruger, 1988b

Table 1.4 - Structural and neurochemical markers of DRG subpopulations (Silverman and Kruger 1988, Silverman and Kruger 1988, Lawson and Waddell 1991, Lawson, Crepps et al. 1997, Zylka, Rice et al. 2005, Brumovsky, Watanabe et al. 2007, Zylka, Sowa et al. 2008, Seal, Wang et al. 2009, Lagerström, Rogoz et al. 2011, Li, Rutlin et al. 2011, Shields, Ahn et al. 2012, Vrontou, Wong et al. 2013)

The subdivision of peripheral afferents by their molecular markers, while diverse, is relatively simpler. For example, the binding of IB4 and FRAP content in non-peptidergic fibers(Silverman and Kruger 1988, Silverman and Kruger 1988), or VGLUT3 in a non-peptidergic LTM C fiber subpopulation(Seal, Wang et al. 2009). The functional relevance of these subdivisions has historically been less clear, with work ongoing to attribute specific sensations to specific fiber populations – largely using ablation techniques.

When considering the role of non-peptidergic nociceptors in peripheral sensation, one of the clearest potential markers for targeting neurones for ablation is IB4 binding, as engaged by Vulchanova *et al* and Tarpley *et al* respectively in studies using IB4 conjugated to saporin (IB4-SAP). Previous work had already suggested that these neurones were involved in nociceptive transmission, though the role in heat transmission appeared to be much smaller than their peptidergic counterparts (Stucky and Lewin 1999), but ablation studies provided a somewhat clearer picture. These IB4-SAP studies suggested a role for non-peptidergic nociceptors in both mechanical and thermal nociception, while similarly pointing to a role for these fibers in mechanical allodynia in neuropathy and thermal hyperalgesia following NGF (Vulchanova, Olson et al. 2001, Tarpley, Kohler et al. 2004). In so doing these studies pointed to a degree of functional distinction between the roles of peptidergic and non peptidergic nociceptors – though later studies raised concerns around the true specificity of IB4-SAP (Cavanaugh, Lee et al. 2009).

Looking further into the discrete differences between molecularly defined receptor sub populations, later studies turned to TRPV1. This polymodal nociceptor has been characterized as localized on a subpopulation of small to medium diameter nociceptors projecting to the superficial layers of the dorsal horn (Caterina, Schumacher et al. 1997, Tominaga, Caterina et al. 1998, O'Neill, Brock et al. 2012). It is worth noting the discrete expression differences between rats and mice – namely that TRPV1 is distributed in both peptidergic and non-peptidergic peripheral afferents in the rat, but that expression becomes restricted to peptidergic fibers in the mouse by adulthood (Cavanaugh, Chesler et al. 2011). Though TRPV1^{-/-} mice demonstrated only a mild reduction in inflammatory thermal hyperalgesia and deficits to thermal nociception above 50°C (Caterina, Leffler et al. 2000), speaking to the redundancy of receptor mechanisms, work in mice in which the entire TRPV1 containing primary afferent population is ablated have characterized insensitivity to noxious heat, a lack of discrimination between 30 and 45 °C in temperature preference assays, failure to develop a Fos signal following 55°C thermal stimulus, and an absence of thermal hyperalgesia associated with either capsaicin or CFA treatment (Cavanaugh, Lee et al. 2009, Mishra, Tisel et al. 2011, Zhang, Cavanaugh et al. 2013). Meanwhile the mice in these studies maintained normal mechanosensation, highlighting the distinct role of this peptidergic nociceptors population in thermosensation and hypersensitivity in mice.

To further investigate the distinct sensory profiles of peptidergic and non-peptidergic nociceptors subpopulations, studies by Cavanaugh and by Zhang have sought to compare the modalities of the TRPV1 positive and MrgprD positive fibers, given this latter population is an exclusively cutaneous, non peptidergic afferent population (Cavanaugh, Lee et al. 2009, Zhang, Cavanaugh et al. 2013). Using behaviour and spinal cord electrophysiology respectively, in Mrgprd^{DTR} mice, intrathecal (IT)

capsaicin treated wild type mice, and IT Capsaicin treated Mrgprd^{DTR} mice these studies demonstrated that:

- MrgprD positive afferents are required for full behavioural sensitivity to noxious mechanosensation and mechanical hyperalgesia, but not thermal nociception; and reduced the evoked responses of superficial dorsal horn NS neurons, with limited effect on the evoked responses of WDR neurons – suggesting a degree of redundancy between the nociceptive mechanical inputs to the deep dorsal horn
- As discussed already, TRPV1 positive afferents are required for behavioural sensitivity to thermal nociceptive stimuli. Further, that loss of this TRPV1 positive population abolishes the evoked responses of superficial and deep dorsal horn neurons to noxious heat
- The loss of both afferent populations was additive, with no further behavioural deficits

Overall, these studies emphasized modality discrimination within the periphery deriving from distinct and non-overlapping peripheral afferent populations. It might be estimated that many more such discreet populations might exist, encoding specific sensory modalities before their integration in the dorsal horn

1.5.1.4 Efferent Function

In addition to the standard transmission of sensory information to the dorsal horn, these peptidergic fibers discussed above also have antidromic function. While the activation of this subpopulation of fibers will lead to the release of both glutamate and neuropeptides, largely in the superficial laminae, in the dorsal horn it also triggers the release of substance P, CGRP and Neurokinin A (NKA) into the periphery.

The effect of these neuropeptides in the periphery is diverse, but ultimately leads to the initiation of neurogenic inflammation through vasodilation, plasma extravasation and immune cell migration(Richardson and Vasko 2002). These peptides bind to their receptors to act either directly on the vasculature or on immune cells to trigger secondary effects, such as mast cell degranulation or leukocyte migration. This is demonstrated by the ability of intradermal administration of these peptides to trigger a wheel and flare response(Hägermark, Hökfelt et al. 1978, Brain, Williams et al. 1985). These peptides similarly have their role to play in the generation of pain, through the activation or perpetuation of the inflammatory cascade, as demonstrated by the attenuation of pain responses in knock out mice(Zhang, Hoff et al. 2001).

Notably, heightened levels of SP are found in the synovial membrane of OA rats(Calza, Pozza et al. 1997), while back-labeling of knee afferents in MIA rats shows knee specific enrichment of CGRP and TRPV1 in the DRG neurons(Fernihough, Gentry et al. 2005), mirroring directly the observations in the clinic(Saito and Koshino 2000). This indicates a potential role for SP, CGRP and TRPV1 in the manifestation of pain in OA, possibly through both contributions to the joint effusions and peripheral sensitization. Electrophysiological studies using peripheral administration of CGRP antagonist BIBN4096BS, in which plasma concentrations never exceeded nanomolar levels, also demonstrated attenuation of WDR neuronal activity following CFA induced inflammation, where spinal drug application had no effect, indicating the importance of peripheral (not central) CGRP in inflammatory pain(Hirsch, Just et al. 2013).

1.5.1.5 Joint Innervation

Morphological analysis of the innervation of the knee indicates that approximately 20% of articular afferents are myelinated, where the majority of this group are A δ fibers, while the remaining unmyelinated 80% are roughly half C fibers, half efferent sympathetic neurons(Grubb 2004). The use of intra-articular tracers confirm that these fibers largely project to levels L3, L4 and L5 DRG/sympathetic ganglia(Edoff, Grenegard et al. 2000), with two major terminal sites in the dorsal horn – Lamina I and Lamina V(Craig, Heppelmann et al. 1988).

1.5.1.6 Spinal terminations of primary afferents

Just as these defined afferent populations show distinct, modality specific functions, these populations also have characteristic termination patterns within the dorsal horn. Broadly speaking the myelinated and lower threshold mechanoreceptive afferent populations innervate deeper in the dorsal horn, from lamina II-V, while the nociceptive populations of A δ and C fibers, notably peptidergic fibers, terminate in the more superficial lamina(Todd 2010).

At a more granular level, work is ongoing to identify where specific subsets of afferents terminate. Using mice designed to express the farnesylated enhanced green fluorescent protein at the TRPM8 locus, Dhaka *et al* demonstrated that TRPM8 containing, and thus cooling sensitive, peripheral afferents terminate in the superficial layer, lamina I(Dhaka, Earley et al. 2008). Using a similar technique, in which placental alkaline phosphatase was targeted to the MrgprB4 locus, Liu *et al* demonstrated that the population of C-fibres innervating hairy skin to convey tactile information terminate in lamina IIo, as part of large arborisations, and co-terminate with IB4 positive fibres(Liu, Vrontou et al. 2007).

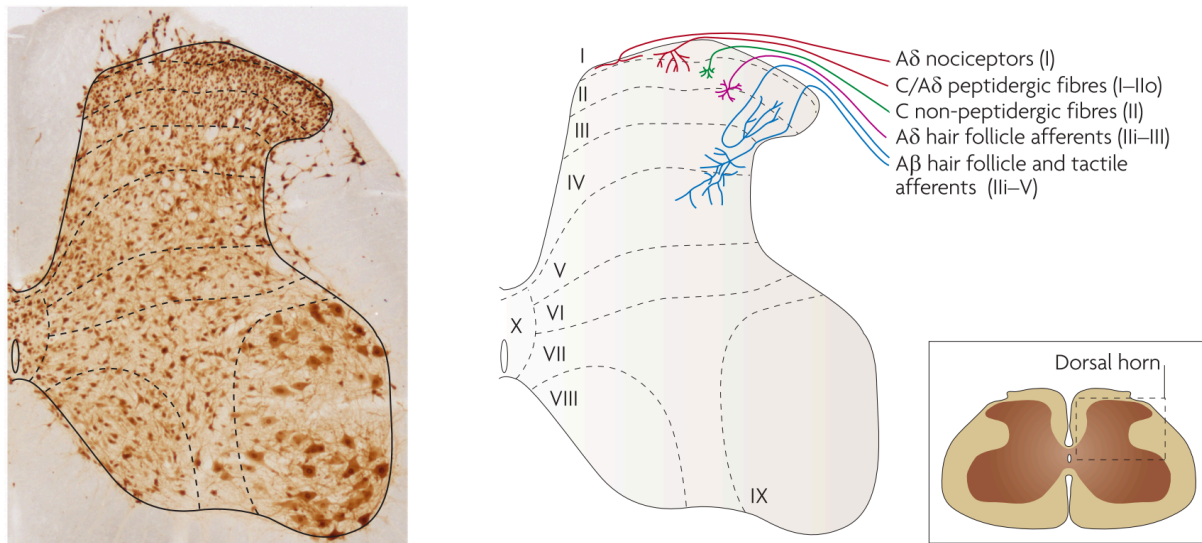


Figure 1.7 – Primary afferent termination in the dorsal horn, taken from Todd 2010 - Left) NeuN immunolabeled rat dorsal horn, demonstrating the laminar boundaries described by Rexed. Note the density of small neurons in lamina I and II. **Right)** Todd 2010 describes a laminar termination pattern based on fiber diameter and function. Namely that Aβ afferents terminate in lamina III–V, with some extension into lamina Iii; Aδ hair follicle afferents terminate at the border of lamina II and lamina III; Aδ nociceptors terminate in lamina I, with some neurones additionally branching to lamina V and lamina X; Peptidergic primary afferents (mainly C, with some 20% of Aδ nociceptors) terminate in lamina I and Ilo, with some divergence into deeper lamina; Non-peptidergic C fibers terminate in the central part of lamina II. (Todd 2010) See the body of text for more details.

However, the location of termination is not the only variable – but the nature of the secondary cell to which the afferent primarily targets. There are considerations of whether projection or interneurons are the post-synaptic target, and given interneurons are most commonly the target (Todd 2010), it is important to consider the considerable segmentation of the interneuron population. For example, the islet and central cells of lamina II will receive direct inputs from C and Aδ, however only C fibers will provide monosynaptic excitatory inputs to these interneurons, while islets received inhibitory inputs from Aδ only (Yasaka, Kato et al. 2007). Meanwhile the C fibers expressing both TRPA1 and TRPV1 synapse to vertical and radial cells of lamina II (Uta, Furue et al. 2010). This peptidergic nociceptor population is also the major synaptic input to NK1 expressing projecting population of secondary order neurones in lamina I (Todd 2002), though these afferent may additionally provide some synaptic input to lamina II and IV (Naim, Spike et al. 1997). The consequence of this segmented input is that the specific ablation of NK1 positive neurones (both projecting and interneuron), using saporin linked substance P, significantly reduces the behavioural shifts associated with inflammatory and neuropathic pain models (Hunt and Mantyh 2001) – as a direct consequence of abolishing a major route of transmission of information from peptidergic nociceptors.

1.5.1.7 Modulating influences on primary afferents

As peripheral afferents progress through the DRG and into the dorsal horn a number of potential interactions may take place before synapsing with the secondary neuron. In experiments using a cre-dependent rabies virus to trace inputs onto primary sensory neurons in neonatal mice Zhang *et al* suggested that sensory neurones likely partook in extra-synaptic regulation of neighbouring afferents within the DRG, likely via Glutamatergic transmission onto the soma(Zhang, Zhao et al. 2015). This is aligned to earlier studies identifying intraganglia release of neurotransmitters(Bráz, Ackerman et al. 2011, Kung, Gong et al. 2013), and potentially represents a mechanism driving the spread of sensitization that contributes to allodynia(Zhang, Zhao et al. 2015). This acts in contrast to the axo-axonic and dendro-axonic GABAergic inputs driving peripheral afferent depolarization (PAD) (Todd, Watt et al. 1996, Kullmann, Ruiz et al. 2005), which ultimately act to reduce the number of action potentials reaching the synapse. Notably, peptidergic nociceptors appear to receive much fewer axo-axonic synapses(Ribeiro - Da - Silva, Tagari et al. 1989). It is suggested that these pre-synaptic interactions can similarly be segmented by fibre type, with Glycine enriched GABAergic input to A δ and A β hair follicle afferents, whereas there is no or limited glycine to the non peptidergic C fibers(Todd, Watt et al. 1996, Watson, Hughes et al. 2002).

The characterization of receptor populations on the terminals of primary afferents in the dorsal horn further speaks to the segmentation of the influences of intraspinal and supraspinal influences. The central terminals of small myelinated A δ and the non-peptidergic C fibers express both 5HT₃ and muscarinic receptors M₂ and M₃(Li, Chen et al. 2002, Zeitz, Guy et al. 2002), while TRPV1 positive, peptidergic populations have been characterized as expressing α_{2A} , 5HT_{1A}, 5HT_{1D}, 5HT₇ and nicotinic receptors(Cardenas, Del Mar et al. 1997, Stone, Broberger et al. 1998, Birder and Perl 1999, Potrebic, Ahn et al. 2003, Haberberger, Bernardini et al. 2004, Doly, Fischer et al. 2005). As such, despite the role of volume transmission in modulating these fiber populations, the discrete expression of specific receptor populations determines the balance of inhibition and excitatory influences exerted.

Recent work by Zhang *et al* has emphasized the predominance of pre-synaptic inhibition of primary afferents by descending GABAergic modulation from the RVM. In this work, the spread of a peripherally injected, cre-dependent rabies virus in advalin-cre P1 pups into RVM cells positive for one of either GLAD2, GlyT2 or Tph2 confirmed these neurones were ~80% GABA/Glycinergic and ~17% serotonergic. Further, the inhibition of this population of RVM neurones suggested that the GABA released by this population is required to suppress hypersensitivity to noxious heat under normal conditions(Zhang, Zhao et al. 2015).

Similarly, Kim and colleagues characterized the importance of descending serotonin for sensitizing the central terminals of primary afferents during nerve injury, via the activation of the 5HT₃ receptor and the facilitation of TRPV1(Kim, Chu et al. 2014). Specifically, in Vc slices from GCaMP3 knock-in, chronic constriction injury of the infraorbital nerve (CCI-ION) mice, treatment with a 5-HT3AR antagonist reduced the capsaicin evoked GCaMP3 signals at the central terminals to levels equivalent to the signals observed in the contralateral side. Similarly, the 5-HT3AR agonist mediated increase in ESPC observed in Vc slices could be partially reversed by pretreatment with a TRPV1 antagonist. As part of a much broader set of experiments, this was taken to suggest that descending 5HT, acting via the 5-HT3AR, recruits and sensitizes TRPV1 to mediate an enhancement of glutamine release at these central terminals(Kim, Chu et al. 2014). This again emphasizes the important role of pre-synaptic regulation of peripheral afferent input to the dorsal horn.

Further discussion of descending controls of spinal cord excitability can be found in Section 1.5.6.

1.5.1.8 Distinct Contributions to Hyperalgesia and Allodynia

As might be expected, given the distinct subdivisions of sensation between peripheral afferents, there is a growing picture of the distinct roles of the fiber subtypes in allodynia and hyperalgesia.

While findings have in part been contradictory, a number of studies have sought to classify the potential role of the A β population in tactile allodynia during inflammation and neuropathy, suggesting that abnormal activity and access of these low threshold mechanosensory fibers to the ascending nociceptive pathways could underlie this pathology. These theories have included the sprouting of A β fibers into lamina I and II(Lekan, Carlton et al. 1996), and a potential phenotypic switch of these fibers to express peptidergic transmitters(Noguchi, Dubner et al. 1994, Neumann, Doubell et al. 1996).

As discussed above, SP expression has been characterized as nociceptors specific characteristic(Lawson, Crepps et al. 1997), however following inflammation Neumann and colleagues characterized the co-expression of SP and GM1 ganglioside in the sciatic nerve DRG using immunohistochemistry and B fragment cholera toxin binding respectively(Neumann, Doubell et al. 1996). The study suggested that this, along side the increased release of SP in the dorsal horn following inflammation, and the increased diameter of SP positive cell profiles after inflammation, suggested a phenotypic switch had occurred in which A β afferents were expressing SP, potentially contributing to the increased excitability of ascending neurones via volume transmission(Neumann, Doubell et al. 1996). A similar suggestion was made by work characterizing increased SP in the

dorsal horn following electrical stimulation of A β fibers after SNL (Malcangio, Ramer et al. 2000). However, more recent work by Hughes has contradicted this, observing neither increased staining for peptidergic transmitters in myelinated fibers, nor internalization of the NK1 receptors when A β fibers are stimulated electrically in various neuropathic models (Hughes, Scott et al. 2003). There is also a suggestion that injured C fibers also bind B fragment of cholera toxin (Shehab, Spike et al. 2003) - similarly serving to contradict earlier work in which had suggested the sprouting of A β fibers after nerve injury (Lekan, Carlton et al. 1996).

While the exact mechanism is unclear, that the abnormal central processing of inputs from this population of low threshold mechanoreceptive A fibers is responsible for allodynia has been demonstrated by intra neural micro stimulation (Torebjörk, Lundberg et al. 1992). Following treatment with capsaicin, the intraneural microstimulation that had previously induced light touch sensations became painful, and A fiber blockade could completely abolish this pain (Torebjörk, Lundberg et al. 1992). Similarly, in a patient lacking low threshold mechanosensitive A fibers, Treede and Cole described the failure of the patient to develop hypersensitivity to light touch following capsaicin injection, despite the usual burning pain, punctate hypersensitivity and flare (Treede and Cole 1993), supporting the role of A β fibers in allodynia during central sensitization.

Using capsaicin and nerve compression to selectively block A fiber conduction, a number of studies have sought to distinguish the distinct roles of different fiber populations during hyperalgesia. Following capsaicin injection, the A fiber block abolished allodynia to stroking, reduced but did not abolish punctate (pricking) mechanical hyperalgesia, and left the “burning” pain unchanged (Torebjörk, Lundberg et al. 1992, Ziegler, Magerl et al. 1999). If a capsaicin pre-treatment was engaged instead, to cause the selective “silencing” of TRPV1+ peripheral afferents (Nolano, Simone et al. 1999), the stimulus response for pinprick was reduced by 32% (versus 82% reduction with A fiber block) while laser evoked pain was eliminated (Magerl, Fuchs et al. 2001). Only when the A block was combined with the capsaicin pre-treatment was sensitivity to pinprick eliminated completely (Magerl, Fuchs et al. 2001).

Combined these studies built a picture in which, during central sensitization, low threshold mechanoreceptive A fibers are the sole transducer of stroke/tactile allodynia, while TRPV1 negative A fibers are the major contributor to pin prick hyperalgesia, with some small contributions from TRPV1 positive C and A fibers. Given this, it is perhaps interesting to note that following CFA induced inflammation the incidence and magnitude of monosynaptic A δ fiber, but not monosynaptic C-fiber, input to lamina I NK1 positive projecting neurons is increased (Torsney 2011). Crucially, these studies suggest that punctate hyperalgesia is facilitated by C fibers, a heterosynaptic

facilitation, but require the activity of A fibers(Torebjörk, Lundberg et al. 1992, Ziegler, Magerl et al. 1999, Magerl, Fuchs et al. 2001, Torsney 2011).

Henrich and colleagues used high frequency electrostimulation, capsaicin pre treatment and A fiber nerve block to look further into the respective roles of C and A fibers in homotropic and heterotropic long term potentiation (LTP), or increased synaptic strength, in the dorsal horn (Henrich, Magerl et al. 2015). This work classified an equivalent role for TRPV1 positive and negative fiber populations in homotropic LTP, while homotropic and heterotropic LTP, observed as secondary hyperalgesia to pinprick, required a much greater contribution of C fibers over A fibers. In other words, this work demonstrated the subdivisions of contribution of different peripheral afferent populations to the generations of increased synaptic strength and the consequential hyperalgesia.

1.5.2 Stimulus Transduction and Transmission

As previously described, afferent endings are either associated directly to stimulus transduction machinery, such as Merkel cells, or exist as free endings in the superficial layers and the joint. These free endings are reliant on receptors, largely ion channels, to transduce mechanical, thermal and chemical information from their environment into depolarizing sensory potentials. These potentials may either directly activate the afferent, by activating voltage gated sodium channels to trigger an action potential, or sensitize the cell, contributing to a shift in the resting potential of the cell to make exceeding the activation threshold more likely. These action potentials are then conducted to the dorsal horn of the spinal cord.

1.5.2.1 Mechano and Thermal Transduction

As the conduction machinery of the lipid membrane, ionophores are crucial components of the transduction and transmission of sensory information. Both inflammation and neuropathy elicit plasticity in the function and expression of these channels, the result of which is the enhanced and spontaneous afferent drive. To date, the full range of somatosensory machinery are as yet fully characterized, but can crudely be broken down into light touch, proprioception, thermosensation and nociception (Gardner, Martin et al. 2000), where nociception itself can also similarly be broken down into thermal, mechanical and chemical.

Across a thermal gradient, 9 different receptors within the Transient Receptor Potential (TRP) family become active and change conformation to increase cation permeability, causing nociceptors depolarization: TRPV1-4, TRPM2, 4, 5 and 8 and TRPA1(O'Neill, Brock et al. 2012). At the most noxious end of the scale, TRPV2 is activated at temperatures exceeding 52°C(Caterina, Rosen et al. 1999), though TRPV1 is believed to be faster acting with a threshold of 42°C(Dhaka, Viswanath et al. 2006).

At the cool end, TRPM8 and TRPA1 are well-characterized transducers of cooling, cold sensation and nociception(Bautista, Siemens et al. 2007, Karashima, Talavera et al. 2009). However the presence of these receptors alone is not sufficient for cold sensation given the inactivation of voltage gated sodium channels by cooling. As such, only Nav1.8 containing afferents, which are insensitive to cooling inactivation, may convey sensory information related to cold and cooling(Zimmermann, Leffler et al. 2007).

Across these thermo-TRPs, there is additional receptivity to a range of chemical and mechanical stimuli. The sensitivity of TRPV1 to pH and capsaicin is perhaps one of the best

characterized(O'Neill, Brock et al. 2012), along side TRPA1 as a polymodal receptor and the “gatekeeper for inflammation”(Bautista, Pellegrino et al. 2013), where both have direct and indirect roles in the sensitization of primary afferents. While TRPA1 is activated by a range of chemical stimuli, including mustard oil, TRPA1 ablated mice exhibit deficits in responses to both low and high intensity mechanical stimuli(Kwan, Allchorne et al. 2006), and TRPA1 antagonist attenuated C fiber activation to high intensity mechanical stimulation(Kerstein, del Camino et al. 2009).

TRPs are becoming increasingly recognised for their role in mechanotransduction, though it is not yet fully understood. TRPV4 is one such candidate. When knocked out this Ca^{2+} permeable osmomechano-TRP channel reduced mouse-tail sensitivity to noxious pressure(Suzuki, Mizuno et al. 2003). TRPV4, which is highly expressed in articular chondrocytes, has similarly been shown to convey load bearing information to allow the regulation of extracellular matrix and joint health, whereby loss of this mechanoceptor is associated with joint arthropathy and osteoarthritis(O'Connor, Leddy et al. 2014).

Attention is also being assigned to the TRPC family of receptors. A study utilizing antisense oligonucleotides showed that TRPC1 and TRPV4 are required for mechanical hyperalgesia but are not required for baseline mechanosensation, whilst TRPC6 plays a role in both mechanical and thermal hyperalgesia(Alessandri-Haber, Dina et al. 2009). Dual knock out of both TRPC3 and TRPC6 silenced a subpopulation of small diameter afferents expressing rapidly adapting mechanically sensitive currents, and additionally resulting in sensory deficits in light touch(Quick, Zhao et al. 2012).

As with cold sensation, the presence of these receptors in the membrane is not enough alone to produce mechanosensation, with increasing support for the importance of Piezo proteins in both noxious and innocuous sensation(Delmas and Coste 2013). Indeed, there are questions as to whether mechanosensation is directly transduced by these channels above, with suggestions TRP may merely modulate or amplify the activity of other unknown mechanosensitive ionophores(Xiao and Xu 2010). In line with this concept of indirect mechanotransduction MrgprD, a G protein coupled ATP receptor, has been proposed to indirectly transduce touch sensation through the detection of ATP release in the skin(Dussor, Zylka et al. 2008).

1.5.2.2 Voltage Gated Sodium Channels

Ionophores also control the electrophysiological properties of the primary afferents, not least the threshold for action potential generation, inter spike intervals and burst duration. Of particular

important to the transmission of pain signals are the voltage gated sodium channels (VGSC) Nav1.3, 1.7, 1.8 and 1.9. Indeed, the differential transmission qualities of the afferent fiber subtypes are in part dependent on the distinct VGSC expression patterns. While A fibers produce narrow, high frequency APs as a result of the predominance of the TTX-S Nav 1.6 and 1.7, C fibers have wider, lower frequency action potential firing patterns due to the predominance of TTX-R Nav1.8 and 1.9.

The importance of these specific VGSC in pain is clearly demonstrated by specific genetic polymorphisms, knock out studies and pharmacology. These will be discussed in more depth in Chapter 6.

In addition to baseline conductance in healthy animals, as has previously been alluded to, these channels are pivotal to the generation of sensitization in the periphery during injury, inflammation and neuropathy. Their currents, thresholds, distribution and inclusion are highly plastic, altering greatly to produce hyperalgesia and allodynia, as discussed below, to contribute to the adaptive pain experience of OA.

1.5.2.3 Ion Channels, Chemical Stimuli and Inflammatory Pain

Following injury and while inflammation is ongoing, the activation of signaling cascades by inflammatory messengers lead to the phosphorylation of VGSC. Alteration in the threshold, current magnitude and kinetics of Nav1.7, 1.8 and 1.9 currents follow, increasing membrane excitability and thus the likelihood that a given stimulus will evoke an action potential(Liu and Wood 2011). This peripheral sensitization can be limited by the use of COX inhibitors, as previously described for OA management, but the effects of these interventions are narrow if the pain is largely neuropathic in nature.

In addition to these post-translational changes to ionophore proteins evoked by the inflammatory milieu, there is dynamic regulation of sodium channel expression, both of their distribution and population. Transcriptional regulation, triggered by both inflammatory cascades and nerve damage, lead to both up and down regulation of VGSC populations. Crucially, following nerve damage there appears to be a renewed expression of Nav1.3, despite restricted expression in normal adult rat DRG(Dib-Hajj, Cummins et al. 2010). It is suggested that this novel expression of Nav1.3 in adult sensory afferents results in ectopic firing and consequently spontaneous pain, symptoms which can be reversed by the application of GDNF which normalizes Nav1.3 expression(Dib-Hajj, Cummins et al. 2010, Liu and Wood 2011).

Similarly, the usually abundant Nav1.8 and 1.9 have been shown to be down-regulated following nerve injury(Dib-Hajj, Cummins et al. 2010, Liu and Wood 2011). However, results are contradictory given alternate studies exhibit a role for Nav1.8 in spontaneous activity in sensory afferents(Roza, Laird et al. 2003). Gold et al argue that while Nav1.8 is down-regulated in the injured neurons, expression is redistributed to uninjured counterparts to produce aberrant activity(Gold MS 2003), which consequently maintains an afferent drive.

Consideration must also be made to the enhanced action of sodium channel populations within sympathetic neurons. Nav1.7 containing sympathetic and sensory neuron populations have been shown to work in concert to enhance pain sensation(Minett, Nassar et al. 2012). While the exact mechanism of a sympathetic drive in acute and neuropathic pain is not yet clear, this system is already being targeted for analgesia in postoperative pain(McDonnell, Finnerty et al. 2011).

1.5.3 Peripheral Sensitization

As previously stated, the onset and maintenance of OA provides considerable activation of the immune cells in and around the joint, producing episodic flares of inflammation as tissue injury progresses. Similarly, the damaged and dying cells (such as the chondrocytes) release distress signals, including ATP(Burnstock 1996), K⁺ and H⁺, which alter the tissue pH(McMahon and Koltzenburg 2006), and prostanoids(Vignon, Balblanc et al. 1993), in and around the joint.

The accumulation of pro inflammatory cytokines in the knee during OA is well characterized(Vignon, Balblanc et al. 1993, Cameron, Fu et al. 1994, Abramson 2004, Gallelli, Galasso et al. 2013). TNF α , the major pro inflammatory cytokine driving cartilage catabolism and degradation(Haseeb and Haqqi 2013), has been detected in 96.1% of patients tested, while 84.6% exhibited IL-6 in their synovium(Vignon, Balblanc et al. 1993). It has additionally been suggested that the levels of IL-6 can be indicative of the stage of OA(Kaneko, Satoh et al. 2000). It is this accumulation of cytokines and inflammatory mediators which is targeted by NSAID analgesia, where treatment with celecoxib, diclofenac or ibuprofen not only produced significant improvement in WOMAC scores, but significant decrease in the IL-6, VEGF and TNF α concentration in the synovial fluid, where higher doses produced better reductions to both criteria(Gallelli, Galasso et al. 2013).

This inflammatory milieu serves dual purposes- not only do these inflammatory mediators directly activate nociceptors, through ligand gated ion channels (LGIC), these mediators can bind to metabotropic receptors, including G-protein coupled receptors (GPCR), to produce sensitization through the initiation of intracellular signaling cascades, many of which converge on common

targets. Consequences are diverse, but largely involve post translational modifications, direct ion channel modulation, altered protein trafficking and expression, and intracellular calcium mobilization. Consequently, there is:

- An observable increase in the response of low threshold A δ afferents to noxious and innocuous stimuli;
- High threshold A δ and C fibers display a decrease in the mechanical stimulus threshold and increase responsiveness;
- The silent, mechanically insensitive nociceptors become mechanically sensitive, increasing input to the spinal cord in response to mechanical stimuli (i.e. fiber recruitment)(Schaible and Schmidt 1985, Grigg, Schaible et al. 1986, Schaible and Schmidt 1988).

These changes significantly contribute to the allodynia and mechanical hyperalgesia observed in OA patients and are termed peripheral sensitization. Outlined below are some example mechanisms of peripheral sensitization by specific mediators involved in OA pain pathophysiology.

1.5.3.1 Activation of LGIC

LGICs are hydrophilic pores in the membrane of the cell whose conformations change from open to closed upon the binding of an agonist. This is the basic mechanism through which neurotransmitters such as Glutamate generate depolarizing potentials to trigger action potentials in neurons. It is similarly the mechanism used by several of these inflammatory agents to shift the membrane potential and sensitize primary afferents. Key transmitter-LGIC relationships in peripheral sensitization include 5HT released from platelets and mast cells at 5HT₃(Sommer 2004), ATP at P2X family receptors, and protons at acid sensing ion channels (ASICs).

To give one example, ATP has been shown to be elevated in OA knees(Ryan, Rachow et al. 1991, Park, Masuda et al. 1996), where it has been characterized that nociceptors (C and A δ) in the knee joint produce rapid, short acting excitation following introduction of ATP or P2X agonist – an effect which may similarly be antagonized(Dowd, McQueen et al. 1998). As such is it is purported by Dowd that the increased presence of ATP in the synovial fluid during the degradation of the joint directly activates these P2X LGIC to contribute to the initiation of nociception and sensitization of nociceptors(Dowd, McQueen et al. 1998). It is of note, however, that these LGIC may have actions on non-neuronal tissues which may contribute to sensitization – where P2X₄ activation on synovial

fibroblasts induces the release of BDNF(Klein, Aeschlimann et al. 2012), whose levels are also elevated in OA(Barthel, Yeremenko et al. 2009).

1.5.3.2 Activation of GPCR

GPCR are a broad family of receptor proteins characterized by a 7-transmembrane receptor that acts in association with a heterotrimer of nucleotide binding proteins. GPCRs are the transducers of a number of messengers, not least 5HT, bradykinin and the prostaglandins. It is this mechanism of peripheral sensitization targeted during NSAID therapy.

These metabotropic receptors drive the excitability of nociceptors during inflammation by the initiation of cascades that result in post translational modifications, largely utilizing either Adenylate Cyclase (AC) and Phospholipase C (PLC).

- **Actions through AC**

For example, the binding of PGE₂ at its EP₂/EP₄, Gs coupled receptor, would stimulate AC, resulting in the production of cAMP. This activates Protein Kinase A (PKA), which phosphorylates serine or threonine residues of receptors and ion channels within the nociceptors to alter their activity. Affected proteins include both those involved in action potential generation, such as Nav1.7 and 1.8, whose threshold and kinetics become altered to increase membrane excitability and thus increase the likelihood that a given stimulus will evoke an action potential(England, Bevan et al. 1996, Gold, Reichling et al. 1996); and receptor proteins like TRPV1, whose phosphorylation results in the reduction in the thermal activation threshold so that the receptor can activate at body temperature, leading to the burning pain associated with inflammation(Moriyama, Higashi et al. 2005).

- **Actions through PLC**

For example, the binding of BK at the B₂ receptor, a Gq coupled receptor, results in the activation PLC. This mediates the liberation of Ca²⁺ from intracellular stores, which may activate Ca²⁺ sensitive ion channels(Gold and Gebhart 2010) or similarly activate Calcium sensitive enzymes, including both Protein Kinase C (PKC) isozymes and Ca-Calmodulin-dependant Protein Kinase II (CAMKII). This can result in both the phosphorylation of nociceptive proteins like TRPV1, whose gating is consequently enhanced(Cesare and McNaughton 1996).

It is worth noting there are multiple points of convergence, explaining the impressive synergy of PGE₂ and BK, which together produce far greater mechanical hyperalgesia than either would alone (Schaible 2009). Similarly, there is considerable redundancy – it is not enough to simply knock out EP₂ to prevent the phosphorylation of TRPV1 during inflammation.

Additionally, while G proteins make considerable contributions to sensitization, they may also provide analgesia. PGE₂ at its EP₃ subtype receptor can provide anti-nociceptive actions, where EP₃ agonist has been shown to reduce neuronal responses of rat knee afferents in inflamed joints, but had no effect in un-inflamed joints (Natura, Bär et al. 2013). It is these endogenous pain control GPCR systems that are utilized by many analgesics, not least opioids.

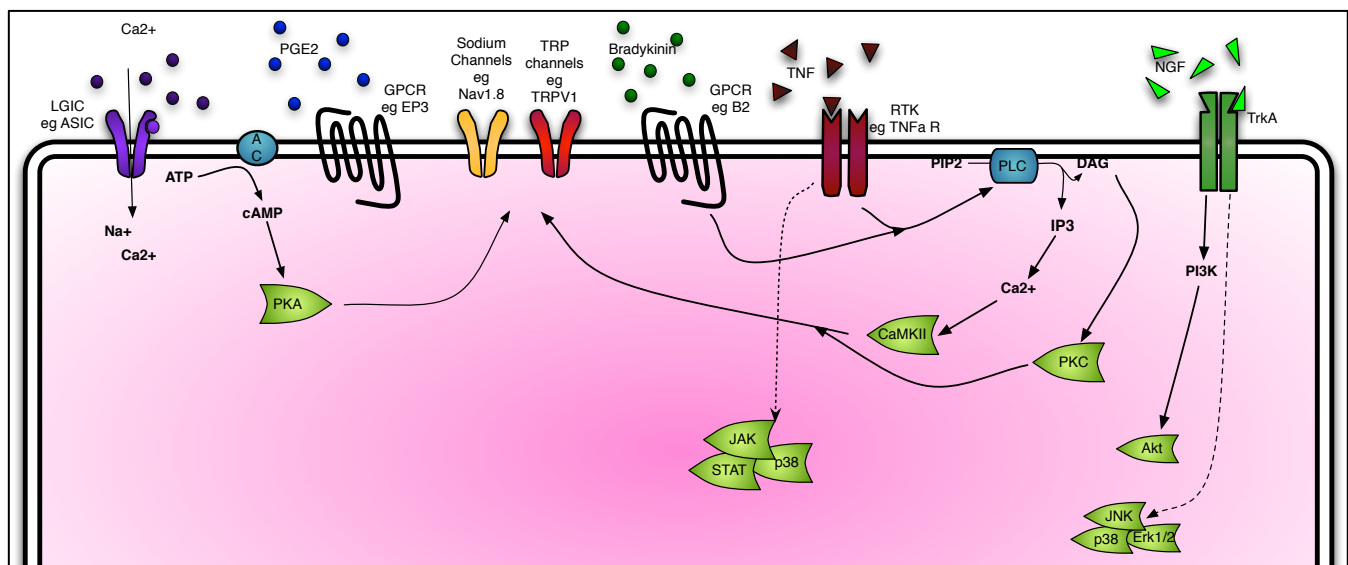


Figure 1.8 - Mechanisms of peripheral sensitization at the peripheral terminal of a sensory afferent | Inflammatory mediators, such as prostaglandin E2 (PGE₂), bradykinin and nerve growth factor (NGF), activate intracellular kinases that phosphorylate transducer channels to reduce their threshold or sodium channels to increase excitability. (LGIC – ligand gated ion channel; GPCR – G protein coupled receptor; RTK – Receptor tyrosine kinase; PKA – Protein Kinase A; AC – Adenylate Cyclase; PLC – Phospholipase C; PKC – Protein Kinase C; CaMKII – Ca-Calmodulin-dependant Protein Kinase II;

1.5.3.3 Tyrosine Kinase Receptor Activation

Receptor Tyrosine Kinases (RTK) are crucial to the generation of peripheral sensitization, as this is the receptor family primarily responsible for the effects of cytokines, where notable examples include TNF-α at p55/60.

These receptors dimerise and recruit p38 MAPK, Janus Kinase (JAK) and Stat transcription factors, to directly alter gene expression and post-translational modification. These receptors also recruit PKC to have more immediate effects on sensory neurons, where IL-1β can produce heat sensitization

within a minute of peripheral injection through its RTK(Sommer and Kress 2004). While targeting these mechanisms, e.g. using antibodies for TNF- α such as infliximab, provide successful analgesia in conditions with a more pronounced inflammatory component, such as Rheumatoid Arthritis (RA), they have little effect in OA, probably due to the smaller role played by inflammation in this condition(Feldmann and Maini 2003).

1.5.3.4 NGF at TrkA

The neurotrophin family, containing both NGF and BDNF, is a critical contributor to peripheral sensitization in inflammation and OA. These mediators were originally characterized for their role in the survival of neurons, but were later recognised for their role in pain and hyperalgesia. The injection of NGF to the Masseter muscle produces prolonged reduction in pressure pain thresholds (PPTs), without alterations in sensations governed by large diameter mechanoreceptive fibers(Svensson, Wang et al. 2008), characteristic of the expression of NGF receptors in peptidergic nociceptors. Similarly, mutations to the NGF receptors have been demonstrated in 3 independent patients with congenital insensitivity to pain(Indo, Tsuruta et al. 1996).

Neurotrophin receptors can be split into two groups: The low affinity p75 receptor, which will bind to any neurotrophin; and the high affinity Trk receptors that bind selectively – NGF at TrkA, BDNF and NT-4 at TrkB, and NT-3 at TrkC. When NGF binds, to cause the dimerization of TrkA, several signaling pathways are recruited, including mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), extracellular signal regulated kinase (ERK) and PLC (Bonnington and McNaughton 2003, Hefti, Rosenthal et al. 2006, Pezet and McMahon 2006). Recruited signaling cascades and their effects include:

- Activation of PI3K, leading to phosphorylation of TRPV1, enhancing its trafficking to the membrane and enhancing the depolarizing current of this channel(Huang, Zhang et al. 2006).
- Regulation of the activity of several transcription factors by NGF-TrkA complex, and its recruited proteins, to alter the expression of a range of nociceptive genes in the DRG neurons, including the peptide transmitters SP and CGRP, ion channels including TRPV1 and Na_v1.8, BK receptors and BDNF (McMahon and Koltzenburg 2006, Ji and Kawasaki 2009).

- Non-transcriptional regulation, altering the rate of mRNA translation through p38 MAPK signaling pathways, allowing for a more rapid increase in expression of nociceptive proteins(Ji and Kawasaki 2009).

As such, NGF not only alters the currents and trafficking of ion channels in the nociceptors membrane, it also alters the expression of nociceptive genes to increase excitability. This acts in concert with the effects of NGF at immune cells, triggering degranulation, cytokine release, proliferation and recruitment.

It has been demonstrated that NGF is increased during OA(Aloe, Tuveri et al. 1992, Walsh, McWilliams et al. 2010), where NGF is released by activated macrophages which have infiltrated the joint. The role of NGF in OA has gained considerable interest following the experimental and clinical success of Tanezumab in mitigating OA pain(Lane, Webster et al. 2005, Lane, Schnitzer et al. 2010, McNamee, Burleigh et al. 2010, Schnitzer, Lane et al. 2011, Brown, Murphy et al. 2012). Unfortunately, this drug has been on clinical hold under the FDA twice since 2010, following reports of serious adverse effects in a minority, not limited to rapidly advancing OA(Garber 2011).

1.5.3.5 Evidence for Peripheral Sensitization in OA

In the context of the joint, peripheral sensitization is observed as pain from previously innocuous joint movements, strain and torque, while noxious stimuli (such as weight placed on an unnatural joint angle) generate greater pain, considered allodynia and hyperalgesia respectively. While OA is not considered an inflammatory disease, the presence of peripheral sensitization is well documented.

Consider first animal work: It has been shown, using single unit electrophysiology, that the induction of OA using the MIA model induces a significant increase in the rate of firing of joint afferents in response to increasing noxious and non-noxious knee torque (Schuelert and McDougall 2006, Schuelert and McDougall 2009); Mechanically sensitive A fibers exhibit marked decrease in their mechanical threshold and increased response to suprathreshold stimuli(Kelly, Dunham et al. 2012); In an acute arthritis model, utilizing carrageenan and kaolin, both nociceptors and non nociceptors showed enhanced mechanosensation, with the additional recruitment of previously silent knee afferents(Schiaible and Schmidt). However, this study by Schiaible & Schmidt 1988 points to limited role of Type II (A β fibers) in the enhanced responses of the arthritic knee, additionally demonstrated by Dorn, Schiaible & Schmidt 1991, where Type II fibers showed normal proprioceptive function in

arthritic joints(Dorn, Schaible et al. 1991). As such, animal evidence seems to point to the predominant role of A δ and C fibers in OA related changes in peripheral sensitivity.

In humans these changes in peripheral fiber excitability are much harder to characterize. The best evidence of the role of peripheral sensitization in OA clinically is the efficacy of anti-inflammatory analgesics, as discussed in Section 1.3.1. These compounds, by blocking the inflammatory cascades responsible for sensitizing peripheral fibers, provide effective analgesia(Zhang, Jones et al. 2004, Towheed, Maxwell et al. 2006, Gallelli, Galasso et al. 2013).

1.5.4 The Dorsal Horn

While pain is initially the product of peripheral sensitization in OA, the sustained sensory barrage produced results in alterations in synaptic processing, termed central sensitization, which make a significant contribution to the overall pain experience during OA(Mease, Hanna et al. 2011). Central sensitization is activity-dependent and characterized by reduced threshold, increased responsiveness and enlargement of the receptive field (Ji, Kohno et al. 2003). In other words, it enhances and facilitates the synaptic transfer of nociceptive information from primary afferents to the ascending neurons in the dorsal horn.

1.5.4.1 Organization

Sensory afferents from the joints enter the spinal cord, via the dorsal root, to synapse within the dorsal horn of L3, L4 and L5(Widenfalk and Wiberg 1989, Ferreira - Gomes, Adaes et al. 2010). It is here that the full range of sensory information is integrated before ascension to the brain. These joint afferents largely project to either Lamina I/II and Lamina V/VI(Sugiura, Lee et al. 1986)[49], where they may form either monosynaptic or polysynaptic connections to ascending neurons and interneurons. Unlike afferent populations, which are heavily sub populated into sensory categories (See discussion in section 1.5.1), these ascending fibers convey a more integrated signal, which is the result of a convergence of multiple afferent sub-types onto common ascending pathways.

1.5.4.1.1 Superficial Layers

Interneurons

The large majority of the superficial layers, laminae I and II, are interneurons – most especially the inner layer of lamina II, which contains a dense population of roughly 95% interneuron, many of which project deeper into the dorsal horn (Spike, Puskar et al. 2003). As such it is perhaps no surprise that the major post synaptic target for the peripheral afferents are the interneurons (A discussion of the peripheral afferent terminations can be found in section 1.5.1.)

The majority of these locally arborizing interneurons are inhibitory (ININs). These cells commonly utilize GABA and/or Glycine – making up approximately 25-40% of the cells of the superficial dorsal horn (Todd and Sullivan 1990, Polgar, Hughes et al. 2003). GABA at the GABA_A receptor appears to provide a tonic level of inhibition, as suggested by the efficacy of the antagonist bicuculline to potentiate NS neuronal responses (Seagrover, Suzuki et al. 2004).

Attempts have been made to sub-classify the ININ populations, where recent work by Polgar et al 2013 propose sub-classification based on the expression of Galanin, NPY, nNOS and Parvalbumin (Polgár, Sardella et al. 2013). These sub-classifications highlight functional differences, for example that nNOS containing ININs upregulated FOS following noxious thermal stimulation or Formalin, but not capsaicin, denoting segregated sensory inputs to these interneurons. They suggest that that nNOS and NPY containing ININs directly inhibit projecting nociceptors to attenuate pain. Perhaps the most widely accepted classifications are as islet, central, vertical and radial cells, which are differentiated by their dendritic morphology (Lu and Perl 2005).

Similarly, a population of excitatory interneurons, expressing vesicular glutamate transporter 2 (VGLUT2) have been characterized. These are directly engaged by primary afferents to transmit nociceptive information to the deep dorsal horn (Millan 2002, D'Mello and Dickenson 2008).

Contribution of interneurons to hyperalgesia and allodynia

Given that interneurons are most commonly the post synaptic target of peripheral afferents (Todd 2010), it is perhaps not surprising that this neuronal population are hypothesized to play a major role in the development of allodynia. Transmission of A β fibre input to the superficial laminae is increased during pain states (Baba, Doubell et al. 1999, Okamoto, Baba et al. 2001, Kohno, Moore et al. 2003, Schoffnegger, Ruscheweyh et al. 2008), an effect which can be replicated by GABA and Glycine receptor antagonists (Torsney and MacDermott 2006, Schoffnegger, Ruscheweyh et al.

2008). Consequently, suggestions center on the concept of disinhibition - the lost inhibition of excitatory interneurons – though studies are in disagreement on whether this is due to a loss of GABA/Glycine, neuronal death or aberrant activity of ININs(Todd 2010).

Candidate interneuron populations include those PKC γ containing EXINs which receive their inputs from low threshold afferents(Miraucourt, Dallel et al. 2007, Neumann, Braz et al. 2008) and have been shown to be the subject of parvalbumin (PV) ININ population modulation, recently termed the gatekeepers of touch evoked pain (Petitjean, Pawlowski et al. 2015). Crucially, within this study Petitjean demonstrated that during neuropathy there are fewer side-by-side positionings of PV and PKC γ containing interneurons in the dorsal horn vs. naïve mice(Petitjean, Pawlowski et al. 2015). Hypothesizing that this might constitute a disinhibition of this EXIN population, Petitjean further demonstrated that intrathecal treatment of these neuropathic mice with a selective PKC γ inhibitor significantly attenuated mechanical allodynia with no effect in naïve mice, while the selective ablation of PV interneurons induced allodynia in naïve mice that could be reversed with a PKC γ inhibitor (Petitjean, Pawlowski et al. 2015) . As such, it seems likely that a loss of inhibition of PKC γ containing EXINs by PV ININs may contribute to the development of mechanical (tactile) allodynia.

Projecting Neurones

The projection neurones of the superficial lamina predominantly project from lamina I, with a limited number arising from lamina II(Todd 2010). As a whole, the lamina is largely considered nociceptive specific, given the nature of the innervation received, however much like the peripheral afferents that activate them, the projecting neurones can also be sub-classified on the basis of their molecular markers and physiological role. For example, using intracellular stains and electrophysiology, Han et al suggested that lamina I populations could be subdivided to include populations of fusiform cells, responsive only to pinch and/or heat; innocuous cooling responsive pyramidal cells; and polymodal multipolar cells, responsive to heat, pinch and cold(Han, Zhang et al. 1998).

Using combinations of retrograde and anterograde tracing the projections and termination of lamina I neurones have been classified as crossing the midline to pass within contralateral white matter to the brainstem and thalamic nuclei, notably to the lateral parabrachial area (LPB), periaqueductal grey (PAG), caudal ventral medulla, nucleus of the solitary tract (NTS) and the thalamus(Burnstein, Dado et al. 1990, Gauriau and Bernard 2004, Todd 2010). As part of these projections there is similarly a large degree of collateralization, allowing projections to multiple nuclei and thus

accounting for the relative proportions of projection destinations as 95% LPB, 33% PAG, 25% NTS and <5% to the thalamus(Al - Khater and Todd 2009).

The consequence of such rich innervation to the brainstem is the capacity for these projecting fibers from lamina I to recruit descending controls (see Section 1.4.5.2). We have previously discussed how the SAP-SP ablation of NK1 projecting neurones attenuated the behavioural hypersensitivities associated with neuropathic and inflammatory pain(Hunt and Mantyh 2001). Interestingly this is an effect replicated by the blockade of descending serotonergic facilitation (Suzuki, Morcuende et al. 2002), further suggesting these projecting fibers recruit descending controls.

1.5.4.1.2 Deep Dorsal Horn

In the deep dorsal horn, at laminae V-VII, projecting afferents receive both direct and indirect sensory inputs – both from the myelinated A fibers from the knee, cutaneous and deeper sources, plus interneurons receiving nociceptive inputs in the superficial layers(Craig, Heppelmann et al. 1988, Light and Kavookjian 1988, Craig 2003, D'Mello and Dickenson 2008). These cells of the deep dorsal horn can be largely grouped into nociceptive specific, as seen in the superficial laminae, and wide dynamic range cells (WDRs) which gain their names through their propensity to fire in a graded manner across a broad range of stimuli(Ritz and Greenspan 1985, Willis 1985). This includes pressure, noxious and innocuous heat and cold.

These WDR cells are the site of a great degree of convergence, since a single cell could theoretically have inputs relating to cutaneous, muscular and joint stimuli(Schaible, Schmidt et al. 1987), which may in part explain the referral of pain(Vecchiet, Vecchiet et al. 1999). A proportion of WDRs then cross over to the anterior spinothalamic tract(Willis, Kenshalo et al. 1979), where they project onward to the thalamus, namely to the central lateral thalamic nucleus, globus pallidus and somatosensory cortex(Carstens and Trevino 1978, Gauriau and Bernard 2004), with additional projections to the PB and hypothalamus(Kitamura, Yamada et al. 1993, Giesler, Katter et al. 1994), suggesting a number of routes are additionally used.

The most notable characteristic of WDR cells is their propensity to “wind up”. This is a form of short-term sensitization where by high frequency inputs of constant intensity evoke increasing outputs from the WDR cell, in contrast to adaptation as one might expect. This is possibly to allow great differentiation by the WDR between innocuous and noxious inputs, since Wind-up may only result from the recruitment of C fibers(Herrero, Laird et al. 2000). This process has been shown to be

NMDA and NK1 receptor dependent(Dickenson and Sullivan 1987, Herrero and Cervero 1996, Herrero, Laird et al. 2000), which is discussed in more detail below (1.4.4.2).

1.5.4.2 The Synapse and Neurotransmitters involved in Transmission

This synaptic transfer of acute nociceptive information from the primary afferents to dorsal horn neurons relies on ionotropic glutamatergic transmission at AMPA and Kainate receptors. In the case of an acute noxious stimulus the membrane depolarization produced will, more likely than not, fail to generate an action potential, instead producing varying degrees of sub-threshold depolarization in the WDR (Li and Zhuo 1998). Prior to the onset of central sensitization, action potentials for noxious inputs are only generated by several EPSPs summing in time or space (Stephen McMahon 2006). Thus, in healthy states, Glutamate acting at AMPA sets a baseline response for noxious stimuli (D'Mello and Dickenson 2008).

However, when these dorsal horn neurons are presented with a barrage of nociceptive information, as in OA, this synaptic transfer is enhanced by a number of distinct mechanism (Stephen McMahon 2006). The first to manifest is Wind-up, defined as the frequency dependent build up of spinal neuronal responsiveness in the WDR neurons(Mendell 1984, Dickenson and Sullivan 1987). High frequency firing of nociceptors results in the co-release of peptide transmitters from nociceptors, including SP and CGRP, which bind to post synaptic GPRCs, most notably SP at NK₁, to produce a slow and building membrane depolarization (De Koninck and Henry 1991, Budai and Larson 1996, Khasabov, Rogers et al. 2002, Suzuki, Hunt et al. 2003). These slow EPSPs, lasting for 100s of ms, readily summate during high frequency inputs to produce a membrane voltage capable of “unleashing” the voltage-dependent Mg²⁺ block of the NMDA Glutamate receptor (Dickenson and Sullivan 1987). This receptor is highly calcium permeable and thus, upon binding of Glutamate, produces a sizable depolarization of the membrane capable of triggering high frequency firing in the WDR, with some help from recruited voltage gated Calcium channels (Stephen McMahon 2006). This activity dependent plasticity presents rapidly during high frequency stimulation (See Figure 1.9) but terminates equally rapidly once the stimulus is removed, thus playing a crucial role in movement evoked pain of OA.

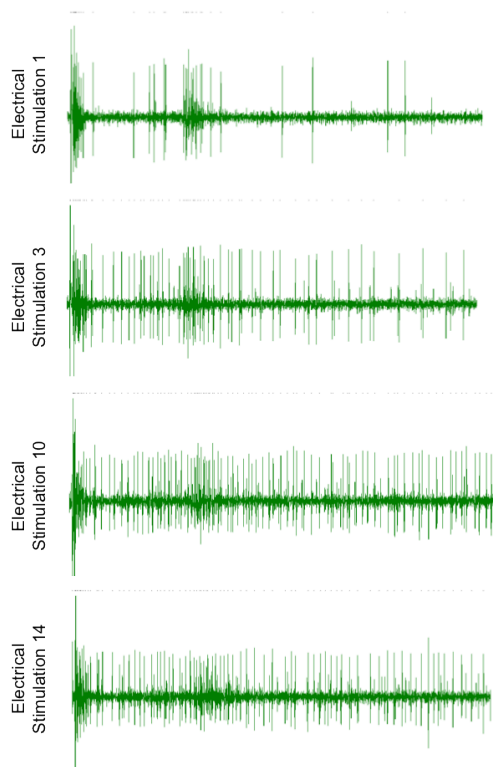


Figure 1.9 - Electrically evoked responses of a Lamina V WDR neuron

Both AMPA and NMDA receptors are involved in the transmission of sensory information in the joint, where NMDA's role is more exclusive to nociception. The application of antagonists for these receptors, CNQX and ketamine respectively, reveal the role of AMPA in both innocuous and noxious sensation but an exclusive role for NMDA in nociceptive sensations from the joint (Neugebauer, Lücke et al. 1993). Ketamine could similarly prevent the development of hyperalgesia during OA, further demonstrating the importance of this LGIC to pain in OA (Boettger, Weber et al. 2010).

Likewise, peptides play a crucial role in nociceptive transmission. The small and medium diameter afferents, most notably those from muscles and joints, utilize the peptides SP and CGRP at both the superficial and deeper dorsal layers (Gibson, Polak et al. 1984, Duggan, Hendry et al. 1988, O'Brien, Woolf et al. 1989, Lawson, Perry et al.

1993). These peptides facilitate the generation of ESPSs in the dorsal horn, as is seen by the ability of co-administration of SP to facilitate NMDA action to facilitate A δ evoked responses and wind up (Chapman, Dickenson et al. 1994), or the reduction of WDR cell responses to noxious and innocuous stimulation of inflamed knees following CGRP antagonism (Neugebauer, Rumenapp et al. 1996).

1.5.4.3 Non-neuronal Contributions to Transmission

As is the case in the periphery (See 1.5.3), the migration, proliferation and release of inflammatory mediators by glia – the immune cells of the CNS – trigger sensitization of neuronal cells. It has been observed that microglia become activated during the development of OA pain, where inhibition of glial cell activation by nimesulide significantly attenuated OA pain (Sagar, Burston et al. 2011). Gibson et al suggest this may in part be due to the loss of microRNA-146a from DRGs in OA, since miRNA-146a acts to regulate and limit inflammatory factors in human glial cells, including TNF α , iNOS and COX-2 (Li, Gibson et al. 2011).

1.5.4.4 Central Sensitization

In addition to wind-up, synaptic processing in the dorsal horn is also subject to the effects of post-translational processing of ion channels, receptors and regulatory proteins, altered expression/trafficking of receptors, and transcriptional changes – much like those seen in the periphery. These changes are part of the process of central sensitization, seen as the expansion of receptive fields, prolonged reduction in threshold and increased responsiveness of neurons (Cook, Woolf et al. 1987, Hylden, Nahin et al. 1989, Woolf 2011). What crucially differentiates central sensitization from peripheral sensitization, since both may result in hyperalgesia, allodynia and spontaneous pain, is the ability of central sensitization to sensitize secondary sites neighbouring the insult (secondary hyperalgesia), to refer pain to entirely unaffected areas, the experience of dynamic allodynia (relating to A β input) and temporal summation (Woolf 2011).

1.5.4.4.1 Central Sensitization: Mechanisms

During pain states, the dorsal horn is bombarded with sensory inputs and awash with glutamate, peptides and inflammatory mediators such as PGE₂ and NGF. The consequence of this is both an increase in intracellular calcium concentrations and activation of GPCRs, such as TrkB by BDNF (Balkowiec and Katz 2000), which lead to the activation of protein kinases (Malinow, Madison et al. 1988, Malinow, Schulman et al. 1989), nitric oxide synthase and ERK (Kitto, Haley et al. 1992, Budai, Wilcox et al. 1995) (Ji, Befort et al. 2002, Kawasaki, Kohno et al. 2004). Specifically, NMDA subunits NR1, NR2A and NR2B become phosphorylated by PKC and Src to remove the voltage dependent Mg²⁺ block and increase open time and kinetics (Zou, Lin et al. 2000, Guo and Huang 2001, Guo, Zou et al. 2002, Brenner, Ji et al. 2004, Woolf 2004), while AMPA receptor GluR1 subunits are phosphorylated by CaMKII to increase single channel conductance (Wang, Wu et al. 2010).

This is accompanied by alterations in the trafficking of glutamate receptors in the synaptic membrane – GluR2 AMPA subunits are internalized, through binding to GRIP, to allow a predominance of higher calcium permeable GluR1 containing receptors, which themselves show increased inclusion (Derkach, Barria et al. 1999, Park, Voitenko et al. 2009, Wang, Wu et al. 2010). There is also an overall increase in the number of AMPA receptors in the synaptic membrane as a result of their increased insertion from intracellular stores (Galan, Laird et al. 2004, Larsson and Broman 2008). These changes alone constitute a significant increase in synaptic efficacy, as can be observed from the ability of knock down or knock-out of GluR1 or NR1 and receptor antagonism to halt the appearance of central sensitization (Chizh, Headley et al. 2001, South, Kohno et al. 2003, Hartmann, Ahmadi et al. 2004).

In addition to these Glutamatergic alterations there are significant changes in gene transcription. The phosphorylation and activation of ERK1 and 2 has both acute effects, such as the direct regulation of potassium currents through phosphorylation of K_v4.2 (Hu, Carrasquillo et al. 2006), or chronic effects, through the activation of cAMP responsive element (CREB)(Kawasaki, Kohno et al. 2004). This leads to the expression of a range of nociceptive proteins, including c-Fos, NK₁ and Trk-B receptors, dynorphin, DREAM and COX-2 (Anderson and Seybold 2000, Ji, Befort et al. 2002, Kuner 2010). Increased transcription of COX-2 ultimately results in elevated PGE₂ in the CNS, which facilitates exocytosis of excitatory neurotransmitter and directly activates ascending neurons (Vasko 1995, Baba, Kohno et al. 2001). As such, the synapses of the dorsal horn become geared towards increased transmission, contributing to the enhanced perception of pain.

Central sensitization also involves a loss of inhibition at a local level. Melzack and Wall, as part of their Gate Control Theory, suggest that loss of this inhibition would result in allodynia in the healthy subject (Melzack and Wall 1965). During OA the PGE₂ produced centrally, as observed in acute rat models of OA(Ebersberger, Grubb et al. 1999), leads to inhibition of glycine receptors containing an α_3 unit (EP GPCR – PKA dependent mechanism) preventing inhibitory signaling by glycine interneurons (Ahmadi, Lippross et al. 2002, Harvey, Depner et al. 2004). As such, there is additionally a loss of inhibition during OA, contributing to the reported hyperalgesia and allodynia.

The feature that critically identifies central sensitization is the development of secondary hyperalgesia. This is the reduction in threshold and increased response to mechanical stimuli in an area neighbouring injury, but not itself damaged. This is clearly demonstrated by the capacity of intradermal capsaicin to induce mechanical hyperalgesia and dynamic tactile allodynia for several hours, even beyond a tight band which prevents local spread of capsaicin or inflammatory mediators(LaMotte, Shain et al. 1991). This is not just a demonstration of clear heterosynaptic facilitation, whereby inputs from C fibers (capsaicin responsive) are conditioning increased responses to A fiber sensory inputs (note that windup is a form of homosynaptic facilitation), but also of the expansion of the receptive field of spinal neurons. As explained by Schaible and Richter 2004 (See Figure 1.7 below), neurons from neighbouring areas may share projecting neurons but under healthy conditions fail to evoke a suprathreshold response. Upon peripheral injury/insults like capsaicin, the barrage of sensory information sensitizes the spinal neuron, lowering the threshold for activation, so that previously innocuous inputs, such as dynamic brush from the silent edges of the receptive field, are sufficient to trigger action potentials – thus expanding the receptive field and accounting for secondary hyperalgesia(Schaible and Richter 2004).

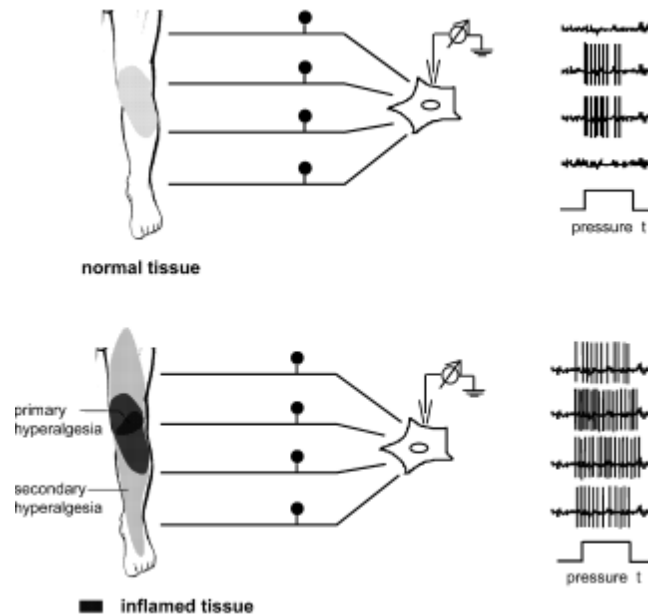


Figure 1.10 - Development of Secondary Hyperalgesia following capsaicin injection, as a consequence of development central sensitization in a spinal cord neuron. Top: Action potentials are generated by stimulation of normal receptive field (shaded region) during healthy state, but not stimulation of surrounding areas from which spinal neuron also receives inputs. **Bottom:** Following insult in the primary receptive field, not only is the elicited response of the spinal neuron greater to the stimulation of the normal receptive field, but the sensitization of the spinal neuron allows generation of action potentials from the neighbouring regions from which it had previously been insensitive. As such the total receptive field expands resulting in a secondary hyperalgesia. (Taken from Schaible and Richter 2004 (Schaible and Richter 2004))

Central sensitization is similarly responsible for the generation of referred pain, as demonstrated by the inability of local anaesthesia and compression block to prevent the generation of referred pain from intramuscular electrical stimulation (Laursen, Graven-Nielsen et al. 1999, Graven-Nielsen and Arendt-Nielsen 2003). However, there still remains a divisions in the field regarding how exactly pain becomes referred. On the one hand, the expansion of receptive fields discussed above may account for pain in healthy tissue. Many similarly cite the likely role of convergence of peripheral inputs, where a co-localization of cells allow a cross talk (Fernihough, Gentry et al. 2004), while synapses to shared ascending cells lead to simple misinterpretation by higher centers regarding the location of the input (Schaible, Schmidt et al. 1987, Giamberardino 2003, Gwilym, Keltner et al. 2009). However, many reject this convergence-facilitation theory for referred muscle pain, stating there is little convergence onto dorsal horn neurons from deep tissues such as joints and muscles (Mense 1994, Vecchiet, Vecchiet et al. 1999), though there may be convergence with cutaneous inputs. It further fails to account for the time delay of referred pain or the stimulus intensity dependence of referral.

Instead referred pain can be attributed to the spreading of the state of central sensitization (Arendt-Nielsen, Nie et al. 2010, Graven-Nielsen and Arendt-Nielsen 2010), which explains both the delay

and the intensity-dependence. This is the idea that the peripheral drive from the joint sensitizes adjacent dorsal horn segments without synapsing to them directly, possibly through interneurons and/or volume transmission. There is additionally a role for descending controls in this referral, discussed later, which creates a facilitated environment in surrounding spinal segments that could account for pain referral.

1.5.4.4.2 Central Sensitization: Evidence in OA

While it is impossible to directly measure changes in synaptic efficacy or evoked responses in patients to demonstrate central sensitization, there are also numerous studies documenting changes in sensory and nociceptive processing in OA patients, which along with the prevalence of referred pain in these patients, point to a CS contribution to OA pain.

Consider resultant pain profiles following the intramuscular injection of hypertonic saline into the tibialis anterior muscle of OA patients and healthy controls. OA patients experienced pain of greater duration and intensity compared to controls, with increased referred and radiating pain(Bajaj, Graven-Nielsen et al. 2001). This muscle hyperalgesia in the absence of peripheral sensitization suggesting that long term nociceptive inputs from the OA knee induce central sensitization in the spinal cord which facilitate messages from the muscle. This would account for the reduced threshold, increased responsiveness and enlargement of the radiating pain area observed in these individuals(Woolf, Thompson et al. 1988, Neugebauer and Schaible 1990, Bajaj, Graven-Nielsen et al. 2001).

Experimenters have similarly demonstrated enhanced temporal summation of pain in patients (an analogue of wind-up), using assessment of pressure pain thresholds across the knee, leg and arms, and impaired diffuse inhibitory controls (DNIC) compared to controls(Arendt-Nielsen, Nie et al. 2010). Sensory profiling of healthy tissue, often using QST, likewise reveals shifts in the sensitivity of OA patients to mechanical stimuli – revealing both reductions in pressure pain thresholds and mechanical hyperalgesia(Bradley, Kersh et al. 2004, Imamura, Imamura et al. 2008, Suokas, Walsh et al. 2012, Wylde, Palmer et al. 2012). Brain imaging studies have shown significantly greater activation in the brainstem of OA patients in response to punctate hyperalgesia compared to controls, including significant Periaqueductal Grey activation, which are considered to be brain biomarkers of central sensitization from previous studies(Zambreanu, Wise et al. 2005, Lee, Zambreanu et al. 2008, Gwilym, Keltner et al. 2009);

While joint replacement resolves OA pain and the related sensory abnormalities for the majority of patients (Kosek and Ordeberg 2000) – highlighting the importance of the peripheral drivers from the joint in OA pain – a minority of patients continue to suffer following surgery, suggesting long term, dysfunctional changes in the transmission of sensory information in the CNS(Wylde, Hewlett et al. 2011). It has been suggested that the failure of joint replacement to resolve chronic pain has a direct relationship to pre surgery severity, whereby patients with more severe symptoms had poorer outcomes(Fortin, Penrod et al. 2002, Lim, Luscombe et al. 2006). This not only reinforces the clinical importance of replacement before it is too late, but also emphasizes the role played by central sensitization in chronic OA pain.

Finally, in one study, lidocaine to one knee in patients with bilateral OA produced a decrease in the VAS scores for both knees(Creamer, Hunt et al. 1996) – indicating that a peripheral drive from one side was contributing to the hyperalgesia of the other through central sensitization.

Alternately, if we look to the animal models of OA they report expanded receptive fields(Thakur 2012), PGE₂ release and COX-2 up-regulation in the spinal cord(Ebersberger, Grubb et al. 1999), increased excitability of ascending neurons(Rahman, Bauer et al. 2009) and a pharmacological efficacy of pregabalin to attenuate mechanical hyperalgesia(Thakur, Rahman et al. 2012), all widely accepted to be indicators of central sensitization. The changes observed in animal models of OA are discussed further in Chapter 3.

1.5.5 Ascending Projections

1.5.5.1 Spinothalamic Tract

The spinothalamic tract is one of the major path through which sensory information ascends from the dorsal horn to the brain, where retrograde tracers injected into the thalamus identify inputs originating in both Lamina I, Lamina V and lamina VII/VIII in primates and cats(Carstens and Trevino 1978, Willis, Kenshalo et al. 1979). These studies also suggested that the majority, ~ 90% of STT cells, ascend contralaterally. Similarly, anterograde labeling in rats, using injections of Phaseolus vulgaris-leucoagglutinin at the superficial lamina, has demonstrated that lamina I neurones project extensively to ventral posterolateral, posteromedial and the posterior group (Po) thalamic nuclei(Gauriau and Bernard 2004).

Attempts have been made to manipulate pain and nociception through either the stimulation or interruption of the STT, causing or blocking burning pain respectively(Craig 2003). However, these

manipulations themselves could result in the development of chronic pain while individual differences in localities of the STT make this an unattractive target for analgesia.

1.5.5.2 Spino-bulbo-spinal Loop

As in the STT, cells project from Lamina I, V and VII in the spinoparabrachial and spinomesencephalic tracts to the brainstem (Cechetto, Standaert et al. 1985, Panneton and Burton 1985, Kitamura, Yamada et al. 1993), to allow the integration of nociceptive information to inform behaviour and homeostasis. While their functional properties and projection paths are similar, unlike the STT these projections are bilateral (Hylden, Hayashi et al. 1986, Kitamura, Yamada et al. 1993), and arise most frequently from Lamina I (Andrew, Krout et al. 2003).

This spinobulbar route terminates in four main regions: the brain stem reticular formation, PB, PAG, and the catecholamine cell groups A1-7 (Hylden, Hayashi et al. 1986, Hylden, Hayashi et al. 1986, Wiberg, Westman et al. 1987, Westlund and Craig 1996). These nuclei have a range of effects including homeostasis, autonomic integrations, emotion, behaviour and continued projection of pain information to the hypothalamus, fore brain and amygdala. Crucially this pathway is part of a loop that projects back to the dorsal horn to provide modulation of spinal excitability, where the tone of modulation is a consequence of both the level of peripheral drive and facilitations or inhibitions from other proximal centers (Kuner 2010).

1.5.6 The Brain, Descending Controls and Pain

The context in which injury and inflammation occur play a large role in determining the extent of the pain we actually experience. This is the direct result of the integration of contextual cues and psychological factors, such as attention and mood, into the processing of nociceptive information in the brain stem. These supraspinal sites contain populations of neurons that project down to the dorsal horn, providing a mechanism for modulating synaptic transmission in the spinal cord (Kwiat and Basbaum 1992, Clark and Proudfit 1993).

The importance of supraspinal influences on pain and transmission in the spinal cord is well documented, with experiments ranging from transections (Danziger, Weil - Fugazza et al. 2001), reversible spinalizations (Schaible, Neugebauer et al. 1991, Herrero and Cervero 1996, Ren and Dubner 1996), electrical stimulations and lesioning of relevant brain regions (Tsuruoka and Willis 1996, Urban, Zahn et al. 1999, Wei, Dubner et al. 1999, Terayama, Guan et al. 2000, Terayama,

Dubner et al. 2002) and pharmacology at both the brain and spinal cord (Green, Lyons et al. 1998, Urban, Coutinho et al. 1999, Green, Scarth et al. 2000, Rahman, Suzuki et al. 2004) confirming their importance in spinal excitability, behaviour and, crucially, the dynamic changes observed following damage and inflammation.

1.5.6.1 Key Pain Centers

1.5.6.1.1 Periaqueductal Gray

The PAG, which quite literally surrounds the midbrain aqueduct, plays a pivotal role in the integration and modulation of pain. As early as 1969 it was acknowledged that electrical stimulation (ES) of the PAG produces profound analgesia (Reynolds 1969), leading to its licensing as a Cancer Pain therapy for a period (Young and Brechner 1986). The PAG feeds into and through the RVM, forming a crucial PAG-RVM pain axis. Microinjection of lidocaine to the RVM has been shown to abolish the effect of PAG ES (Sandkuhler and Gebhart 1984), demonstrating the importance of the RVM as the relay point for PAG controls on pain.

This PAG-RVM axis acts as an integration site for inputs from various brain regions with those directly ascending from the superficial laminae (Hylden, Hayashi et al. 1986, Kuner 2010), providing the basis for the role of context on descending controls. Neurons feed into the PAG from areas including the Amygdala, Hypothalamus, Frontal Lobe and the Anterior Cingulate Cortex (ACC) to modulate activity based on attention, emotion, stress and setting (Lovick 1993, Bandler and Shipley 1994, Ossipov, Dussor et al. 2010). These contributions set the level of input into the nucleus raphe magnus (NRM) and nucleus gigantocellularis (NGC) before these neurons descend to the dorsal horn via the dorsolateral and ventrolateral funiculi. In addition, the PAG and RVM communicate with the Noradrenergic nuclei, most notably the locus coeruleus (LC), A5 and A7, recruiting them to provide their own direct noradrenergic modulation in the dorsal horn (Holden and Proudfit 1998, Bajic and Proudfit 1999).

Interestingly, the PAG has been shown to differentially modulate myelinated vs. unmyelinated fibers inputs. Specifically, descending inhibition from the rostrocaudal extent of the dorsolateral/lateral and ventrolateral columns of the PAG preferentially target WDRs with C fiber inputs (McMullan and Lumb 2006, Waters and Lumb 2008). The functional implication is that PAG may limit the slower, less well localized burning pains conveyed by C fibers, and could in part be important in limiting the effect of wind up in these WDR cells, given this summation is similarly C fiber dependent.

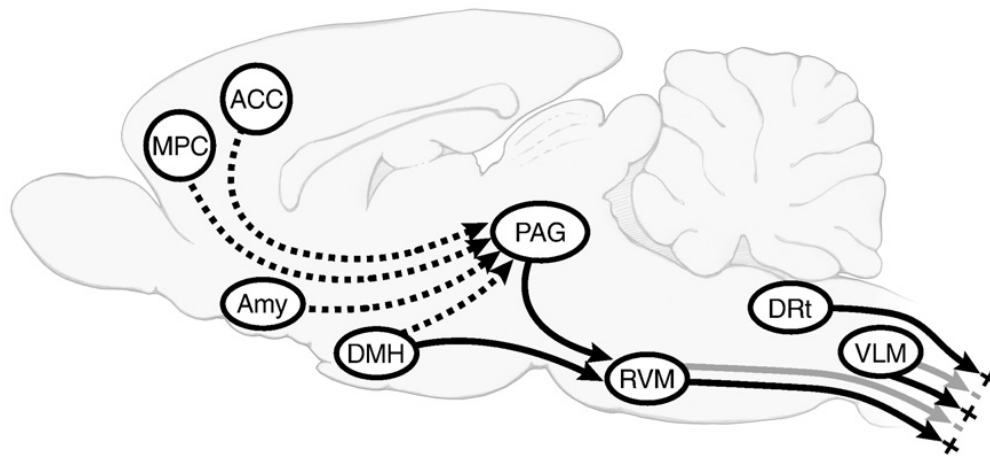


Figure 1.11 - Schematic diagram of descending controls in the rat brain: The Periaqueductal Gray (PAG) receives direct and indirect inputs from limbic forebrain areas including anterior cingulate cortex (ACC), amygdala (AMY), dorsomedial nucleus of the hypothalamus (DMH), and medial prefrontal cortex (MPC). This then feeds into the Rostral Ventromedial Medulla (RVM), which additionally received inputs from DMH, and descends to the dorsal horn to exert bidirectional control. DRt and VLM additionally receive these inputs (not shown), where DRt is thought to be facilitating, and VLM primarily inhibitory. **(Taken from Heinricher 2009 (Heinricher, Tavares et al. 2009))**

1.5.6.1.2 Rostral Ventromedial Medulla

It is now very well established that neurons descend from the Rostral Ventromedial Medulla (RVM) to modulate transmission in the dorsal horn, with converging evidence highlighting the ability for these controls to either enhance or diminish nociceptive processing (Fields, Basbaum et al. 1977, Fields, Bry et al. 1983, Mokha, McMillan et al. 1985, Mokha, McMillan et al. 1986, Heinricher, Barbaro et al. 1989, Heinricher, Morgan et al. 1994, Ren and Dubner 1996, Wei, Dubner et al. 1999, Burgess, Gardell et al. 2002, Neubert, Kincaid et al. 2004, Bee and Dickenson 2007, De Felice, Sanoja et al. 2011). This dichotomy of function is best observed in studies using electrostimulation (ES) and microinjections of Glutamate to the RVM – where small doses of Glutamate or low intensity ES produces facilitation in the dorsal horn while high dose Glutamate or high intensity ES inhibits transmission (Zhuo and Gebhart 1992, Zhuo and Gebhart 1997).

Evidence suggests that both inhibitory and facilitatory systems are activated during acute nociception but that the discrete balance between these outputs may change over time to determine the extent of nociceptive transmission and pain experienced (Porreca, Ossipov et al. 2002, Vanegas and Schaible 2004). Lesioning studies have suggested that while the NRM is responsible for descending inhibition of spinal excitability, where lesioning enhanced thermal hyperalgesia in the first 24hrs of inflammation, this is counterbalanced by the NGC, which provides descending facilitation and whose lesioning entirely attenuated thermal hyperalgesia (Wei, Dubner et al. 1999). Crucially, the discrete balance between these opposing systems is dynamic and shifts during the time

course of injury/disease, as well as differing depending on the extent and kind of insult (**See 1.4.6.2**).

This bi-directional control of pain is observed in the firing patterns of 3 distinct classes of cells found within the RVM: ON cells, OFF cells and Neutral cells (Fields, Bry et al. 1983, Heinricher, Barbaro et al. 1989). ON cells increase their firing just prior to the initiation of nociceptive reflex (such as tail flick in the rat) while OFF cells are tonically active and decrease firing prior to nociceptive reflexes. Neutral cells show no real change in activity prior to or during the nociceptive reflex. Crucially, the activity of ON and OFF cells has been linked to the facilitation and inhibition of pain transmission respectively. Thus during periods of increased OFF cell activity there is an observable increase in latency to tail flick and conversely increases ON cell activity shortens this latency (Barbaro, Heinricher et al. 1989, Heinricher, Barbaro et al. 1989).

The key characteristic of the RVM and these ON/OFF cells is their role in opioid analgesia. The administration of morphine causes significant reduction in the firing of ON cells while enhancing the activity of OFF cells (Heinricher, Morgan et al. 1994). During normal/acute pain states this activation of OFF cells is both necessary and sufficient for opiate analgesia, while inhibition of ON-cells alone is insufficient (Fields, Basbaum; et al. 2006). However, during chronic pain states such as inflammation the direct opioid inhibition of ON cells has a much greater impact on hyperalgesia (Porreca, Ossipov et al. 2002), a result of the increases activity of ON-cells during inflammatory pain.

Controls descending from the RVM utilize 5HT (Serotonin) as the major transmitter by which they control excitability, along side some GABA and Glycine (Kato, Yasaka et al. 2006) and Noradrenaline (NA) from the LC to modulate spinal excitability. These transmitters bind to the presynaptic boutons of the primary afferents to affect neurotransmitter release or bind to post synaptic sites on secondary or ascending neurons to modulate excitability and firing. While NA produces a direct inhibitory effect on neurons through α_2 adrenoceptor (Millan 2002), 5HT can be either inhibitory or excitatory, depending on the receptor subtype. Dogrul and colleagues demonstrated that serotonin at 5HT₇ will inhibit transmission and provide analgesia while 5HT₃ enhances transmission to induce/sustain hyperalgesia (Dogrul, Ossipov et al. 2009).

While the role of enhanced or diminished serotonergic controls in the dorsal horn in acute, inflammatory, and neuropathic pain conditions are well confirmed by pharmacology (Green, Scarth et al. 2000, Suzuki, Rahman et al. 2004, Suzuki, Rygh et al. 2004, Rahman, Bauer et al. 2009, Sikandar, Bannister et al. 2012, Burnham and Dickenson 2013, Wang, King et al. 2013), it is suggested that these dynamic changes may need a sufficiently large peripheral drive to cause a shift. For example, a model of carrageenan inflammation did not alter levels of descending facilitation from those seen in

naïve animals but facilitation was enhanced in both stages of the formalin response, as revealed by the 5HT₃ antagonist ondansetron (Green, Scarth et al. 2000, Rahman, Suzuki et al. 2004). This is a reasonable suggestion if we consider descending control to be a form of brain stem sensitization, as is suggested by Miki (Miki, Zhou et al. 2002). As in the dorsal horn, where central sensitization is a frequency dependent build up in responsiveness, it may be that the intervention of descending controls is frequency dependent, relying on a certain level of ascending input. This has been verified by Suzuki and colleagues, who demonstrated that the analgesic effect of selective ablation of NK₁-containing ascending neurons could be reproduced by ondansetron during the second phase of formalin response (Suzuki, Morcuende et al. 2002). Thus these ascending NK₁ containing neurons of lamina I/III are driving descending, serotonergic controls

This bi directional control of pain by the RVM presents an interesting target for pain control, given the position of the discreet balance will determine the overall pain experience. It is similarly of considerable interest to define where the balance lies during chronic pain conditions such as OA, to help better our understanding of this condition. Preliminary work has suggested there is an increased descending serotonergic facilitation during an MIA model of OA (Rahman, Bauer et al. 2009), but how this changes over time and with disease severity is as yet unclassified.

1.5.6.1.3 Dorsolateral Pontine Nuclei

The dorsolateral pontine (DLP) noradrenergic cell groups are a rich source of NA in the spinal cord, most especially from the locus coeruleus (A6), A5 and A7 nuclei (Westlund, Bowker et al. 1983, Westlund, Bowker et al. 1984, Kwiat and Basbaum 1992), where projections appear to largely terminate in the deeper laminae of the dorsal horn (Clark and Proudfit 1991). It is here that the release of noradrenaline provides inhibition of spinal excitability – as has been well characterized by the spinal application of NA (Engberg and Ryall 1966, Headley, Duggan et al. 1978). Similarly, the application of NA antagonists has been shown to be hyperalgesic (Sagen and Proudfit 1984), including reversing the analgesic effects of systemic morphine (Proudfit and Hammond 1981).

As in the PAG, the ES of the DLP produced strong inhibition of tail flick in lightly anaesthetized rats, an effect most potent when directly stimulating the LC (Jones and Gebhart 1986). This descending inhibition was effectively blocked by the intrathecal introduction of α_2 receptor antagonists Yohimbine, pointing to a α_2 mediated noradrenergic inhibition descending from the DLP (Jones and Gebhart 1986). This anti-nociceptive and anti-hyperalgesic control of spinal excitability by the DLP/LC is now very well characterized (Segal and Sandberg 1977, Jones and Gebhart 1986, Mokha,

McMillan et al. 1986, Jones and Gebhart 1988, Clark and Proudfit 1991, Clark and Proudfit 1993, West, Yeomans et al. 1993).

It is suggested that nociceptive inputs activate descending inhibition, where the extent of the inhibition is both intensity dependent, surmountable and dynamic over the time course of injury (Stanfa and Dickenson 1994, Tsuruoka and Willis 1996, Tsuruoka and Willis 1996, Green, Lyons et al. 1998, Tsuruoka, Hitoto et al. 1999, Malmberg, Hedley et al. 2001, Molina and Herrero 2006). It is apparent across the breadths of these studies, which utilize lesioning, knock out animals and pharmacology, that this descending inhibition requires “switching on” as it is not active in naïve, uninjured animals - with the exception of responses to high intensity noxious inputs in naïve animals (such as tail flick). This is a pain protection system that becomes switched on by the barrage of nociceptive information and as such is an attractive target for clinical pain management. Hughes et al 2013 suggest that this descending noradrenergic system spatially restricts and temporally delays the expression of neuropathic pain, but loses influence once neuropathic pain is established – as seen by the lost effect of α_2 receptor antagonism and using this to explain the lost efficacy of NA therapies in neuropathic pain (Hughes, Hickey et al. 2013). The suggestion is that neuropathic pain may be better managed by earlier interventions with therapies designed to manipulate NA systems, notably SNRIs and tricyclics, before descending noradrenergic inhibition loses its influence. This has already been effective in the prevention of progression of shingles to post herpetic neuralgia (Bowsher 1997), and the implications of this to the management of advanced OA pain could be interesting.

1.5.6.2 Changes to Descending Controls during Inflammation

While changes to descending controls and supraspinal processing in OA are as yet not fully classified, it is perhaps most informative to consider the changes in these descending systems during acute and chronic inflammation.

Initiation of Inflammatory Pain

It has been shown that during the initial hours of inflammation descending controls gear towards facilitation. Terayama and colleagues showed that at 3hrs after the induction of inflammation there is a rightward shift in the ES stimulus–response curve compared to 1hr time points (Terayama, Guan et al. 2000). In other words, a greater ES is required to gain the same increase in paw withdrawal latency (PWL), indicating a shift in the balance of descending control towards facilitation, where this shift is shown to relate to plasticity of the NMDA receptor population (Terayama, Dubner et al. 2002). This

shift was not observed when ES was applied directly to the dorsolateral funiculus, indicating that the change is located in the RVM and not at the spinal level. These studies suggest the predominance of facilitation during the onset of inflammatory pain, driven by NMDA receptor sensitivity in the RVM.

It is proposed that this initial facilitation originates within the NGC, a site previously shown to be a point of origin of descending facilitation (Zhuo and Gebhart 1992, Wei, Dubner et al. 1999). Consequently, lesioning of the NGC reduces the current intensity of ES to the RVM required to produce complete inhibition of inflammatory hyperalgesia (Terayama, Dubner et al. 2002). It has additionally been shown that molecular depletion of 5HT from these NGC neurons attenuates the development of mechanical hyperalgesia and allodynia after CFA injection, suggesting serotonergic neurons have a significant role in facilitating the development of hyperalgesia following inflammation (Wei, Dubner et al. 2010).

Given this information, it seems feasible that in the initial hours of inflammation the sensory barrage ascending to the brain stem activates facilitatory descending controls. As with peripheral sensitization, this early facilitation is beneficial as a mechanism for limiting the use of injured tissue.

Modulation of Established Inflammatory Pain

It is now established that during inflammation, beyond this initial facilitation on day 1, there is a superseding shift towards descending inhibition of the site of primary hyperalgesia, which acts to limit the impact of accumulating peripheral and central sensitization. First suggested by Schiabe and colleagues, who reversibly spinalized cats using cooling of the spinal cord, it was shown that inflammation induced a progressive enhancement of descending inhibition (Schaible, Neugebauer et al. 1991). This effect was similarly replicated using lidocaine to block descending controls during CFA induced paw inflammation (Ren and Dubner 1996).

Terayama and colleagues showed that this predominance of inhibition does not occur immediately, rather developing after an initial period of facilitation. After the initial increase in current intensity required for complete inhibition of PW observed at the 3rd hour of CFA inflammation there is a decrease over the next 21 hours, shifting the stimulus response curve to the left, indicating the switch to a net descending inhibition (Terayama, Guan et al. 2000, Terayama, Dubner et al. 2002). This leftward shift is similarly replicated in NMDA and AMPA dose-response curves 24hrs post inflammation (Guan, Terayama et al. 2002). This is indicative of a switch in the RVM to descending inhibition, originating from plasticity at glutaminergic synapses (Vanegas 2004). This plasticity similarly increases the sensitivity of the RVM to opiates (Zhang and Hammond 2010).

Single unit recordings from the RVM similarly support the idea of a shift towards inhibition from the RVM(Miki, Zhou et al. 2002). Continuous recordings over the 3-6 hours after CFA identified a phenotypic switch of neutral cells to pain modulating on-like or off-like cells, as was not seen in naïve animals. This was confirmed with a population study. There was similarly a reduction in the number of off-like cells showing a pause of activity after noxious stimulation after inflammation, which together lead to a suggestion that RVM neurons may switch to favour descending inhibition(Miki, Zhou et al. 2002).

This enhancement of descending inhibition is not limited to the RVM however but involves the noradrenergic system too. Using lesioning, Tsuruoka et al suggests that inflammation activates inhibitory controls originating from the LC to restrict the development of hyperalgesia during inflammation(Tsuruoka and Willis 1996). However, the effect of lesioning are lost by the 7th day of inflammation, whereby no difference is observed between the hyperalgesia between the sham and lesion groups(Tsuruoka and Willis 1996). This suggests that the LC and NA are only involved in descending inhibition during a short initial window of inflammation, as is observed in both the MIA model of OA and tibial nerve injury neuropathic pain model(Hughes, Hickey et al. 2013).

In considering the plasticity of these systems other experiments have shown that, much like in the dorsal horn, there are significant alterations in gene expression and receptor populations over the course of inflammation. Miki and colleagues, in proposing the concept of brainstem sensitization, identified peripheral inflammation induced changes in NMDA receptor gene expression in the RVM (Miki, Zhou et al. 2002). They identified a significant increase in NMDA subunit mRNA over the proceeding 1-7days after CFA injection, with the greatest increase in NR2A unit mRNA and protein. Similarly, inflammation induces a significant increase in AMPA receptor subunit mRNA, with significant up-regulation of GluR1-flip protein over 24hr-3days after CFA(Guan, Guo et al. 2003). It is well established that Glutamate plays a prominent part in excitatory transmission in the RVM and activation of descending control from brainstem sites(Aimone and Gebhart 1986, Beitz 1990, Spinella, Cooper et al. 1996), where we have discussed above the shift in the dose response curves for this transmitter during inflammation(Guan, Terayama et al. 2002). As such, these results would suggest that part of the increase in descending control observed during inflammation may originate from an increase in NMDA and AMPA receptor populations, composed of subunits with high conductance properties (NR2A) and a reduced rate of desensitization (GluR1-flip), which increase excitability and activation of RVM neurons. Since the activation of AMPA receptors in the RVM mediate descending inhibition(Urban, Coutinho et al. 1999), the growth of this receptor population in the RVM during the first week of inflammation goes some way to explain the increased descending inhibition observed to be limiting acute inflammatory pain. However, the leftward shift in the dose response curve of AMPA and NMDA in descending inhibition was significant as early as 5hrs after the induction of

inflammation when these protein changes only reach significance after 24hrs(Guan, Guo et al. 2003), pointing to additional, faster acting mechanisms of plasticity in the brain stem.

The complexity of the overall pain profile during inflammation is added to by evidence suggesting that primary and secondary sites of hyperalgesia may be differentially controlled(Vanegas 2004). It is suggested that there is an inhibitory drive to the area of primary hyperalgesia, but a facilitatory drive in the surrounding spinal segments that underlie secondary hyperalgesia and referred pain. This is demonstrated by the ability of lidocaine, NMDA receptor or neurotensin receptor antagonists in the RVM to attenuate the development of secondary thermal hyperalgesia during paw inflammation(Ren and Dubner 1996, Urban, Coutinho et al. 1999, Wei, Dubner et al. 1999). On the basis of this evidence we would expect a descending facilitation of noxious transmission from areas of secondary hyperalgesia, for example the rat paw in a model of knee OA, and inhibitory controls presiding over the joint itself.

When we consider OA specifically, much is yet to be understood about the role of supraspinal controls in OA pain. While some work has been done to characterize how these influences may change during OA, and the consequences this may have on OA pain, the picture is by no means complete. Early work using either cold block spinalizations or transection to elucidate the role of descending controls in inflammatory joint pain revealed the increase in descending inhibition which followed in the initial 24hrs(Schaible, Neugebauer et al. 1991, Danziger, Weil-Fugazza et al. 1999). Brain imaging has similarly suggested greater activation of the PAG in OA patients receiving punctate stimulation to areas of referred pain vs. controls(Gwilym, Keltner et al. 2009), suggesting these spinally projecting brain stem centers may be highly relevant to the generation of the overall OA pain profile. More recently, work from this lab has characterized the chronic shifts in descending controls during the MIA model of OA. They showed adaptive changes in serotonergic controls that may underlie increased evoked responses to dynamic brush and innocuous punctate stimuli(Rahman, Bauer et al. 2009), along side work revealing a time sensitive effects of atipamezole or milnacipran plus atipamezole on evoked responses in the early stages of MIA induced OA(Burnham and Dickenson 2013). These fall in line with expectations provided by inflammatory pain models. We would expect a prolonged descending facilitation of noxious transmission from the paw to maintain a secondary hyperalgesia in OA, as well as a time sensitive noradrenergic inhibition of transmission, which resolves by day 7. However, questions remain about how these findings may change in a lower dose MIA model, or what may be observed regarding serotonin in the earlier stages.

1.6 Thesis Aims

For patients with symptomatic OA the unmet needs are still clear – efficacious, safe and tolerable analgesia. If we are to address these to provide superior quality of life for patients, it is crucial that we continue to expand our knowledge of the mechanisms underlying pain during OA so we can better manipulate and target these mechanisms to mitigate their impact.

The experiments described in this thesis aimed to:

- Characterize differences in the pain phenotype and extent of descending control, both serotonergic and noradrenergic, in the early and later stages of a 1mg MIA model of OA pain versus previously published work in a 2mg neuropathic model of OA pain, using behaviour and spinal cord electrophysiology; And relate these differences and the pharmacological implications back to the clinic.
- Identify whether a low dose MIA model of OA pain induced adaptations in the response properties of the pain responsive cells of the RVM using electrophysiology.
- Evaluate the role of a specific sub-population of sensory afferents and a population of mechanosensory receptors in the development of pain during OA in mice.

Chapter 2 – Methods

All procedures were approved by the UK home office and followed the guidelines of the International Association for the Study of Pain (Zimmermann 1983).

2.1 Animals

2.1.1 Rats

All work was conducted in Male Sprague Dawley rats, bred and housed in the Central Biological Services Unit at University College London. Experimental animals can be split into six distinct groups:

- **Arthritic:** Those animals in which the OA pain state was induced by the injection of MIA. For spinal electrophysiology, these animals were investigated either at the early stages of OA pain development, at days 3-5 after injection, or late stages, at days 10-14 after injection. For RVM electrophysiology, animals were investigated 14-16 days after injection. Induction weights differed to ensure behaviour and electrophysiology was conducted in animals of the same weight and age, in the range of 220-250g for spinal electrophysiology, and 250-300g for brain electrophysiology:
 - **Early:** MIA injection into animals weighing 160-180g.
 - **Late:** MIA injection in animals weighing 120-140g.
 - **Brain Recording:** MIA injection into animals weighing 160-180g
- **Shams:** Those animals in which OA was not induced but instead received an intra-articular injection of Saline. Sham animals were investigated across the same time scale, to track time dependent changes resulting from the procedure itself. These animals were similarly age and weight matched so that all behaviour and electrophysiology was conducted in animals of the same weight and age, in the range of 220-250g for spinal electrophysiology and 250-300g for brain electrophysiology.
 - **Early:** Saline injection into animals weighing 160-180g.
 - **Late:** Saline injection in animals weighing 120-140g.
 - **Brain Recording:** MIA injection into animals weighing 160-180g

Animals were housed at a maximum of five per cage with *ad libitum* food and water, on a 12hour day and night cycle.

Naïve controls, where used, were procured directly from BSU stock at a weight in the range of 220-250g.

2.1.2 Mice

Work was conducted in male and female mice from four different genetic lines, all of which were bred and housed in the Cruciform Biological Services Unit at University College London. Animals were housed as a maximum of 6 per cage, with *ad libitum* food and water, on a 12hour day and night cycle. Mice aged 6-8weeks were used for induction of OA pain state, induced by intra-articular injection of MIA.

Mouse Lines:

- **“DTA Mice”:** This mouse line was generated by crossing heterozygous Nav1.8 Cre mice with homozygous eGFP-DTA mice. This generated a litter of half controls (wild type, WT) and half DTA mice, where DTA mice have all the post-mitotic sensory neurons containing Nav1.8 eradicated through the expression of diphtheria toxin A(Ivanova, Signore et al. 2005, Stirling, Forlani et al. 2005, Abrahamsen, Zhao et al. 2008). A full sensory profile is described in Abrahamsen et al 2008.
- **TRPC knock out mice:** Quick et al 2012 generated three mouse lines for use(Quick, Zhao et al. 2012):
 - **TRPC3 SKO**
 - **TRPC6 SKO**
 - **TRPC3/6 DKO**

A double knock out mouse had originally been generated by Birnbaumer and colleagues at the NIEHS. Their DKO mice were crossed with C57BL/6 mice to create heterozygous TRPC3^{+/-};TRPC6^{+/-} mice. These could then be crossed together to create DKOs, single KOs and WT controls. A full sensory profile is described by Quick et al.

2.2 Induction of OA

2.2.1 Rats

The dose selected for this protocol was based upon findings of Thakur et al 2012, which described differential pain profiles between 1mg and 2mg MIA rats. Previous work in this lab had already characterized descending controls for the 2mg profile, so we sought to characterize this less severe, non-neuropathic 1mg MIA model(Thakur, Rahman et al. 2012).

Male Sprague Dawley rats, weighing either 160-180g (early group) or 120-140g (late group), were anaesthetized using 3.5% isoflurane in a 2:1 mixture of Oxygen and Nitrous Oxide until animals were a-reflexive. Animals were then placed on their backs, on a heat mat, and maintained on a nose cone at 2% isoflurane. The absence of reflexes was re-assessed. The entire ventral surface of the hind limb, from paw up to mid thigh, was then clipped of hair and cleaned using clorhexidine. This both minimized the risk of infection but rendered the skin more soft, malleable and translucent for visualizing the injection site. Reflexes were checked one final time before OA was induced by injecting 1mg MIA in 25µl of 0.9% saline into the left knee of the flexed joint. The MIA was administered using a 27G needle through the left patellar tendon, which was held in place for 30s and withdrawn slowly to minimize leakage. The limb was then flexed and extended a few times to distribute MIA, re-cleaned and the animal placed in an incubator to recover. Sham animals received saline injection only. Once animals had recovered they were returned to their cages and monitored over the proceeding 24-72 hours by trained staff in the animal unit. The injection day is considered day 0 for the subsequent studies.

2.2.2 Mice

The protocol for establishing OA in mice using MIA is less well established, at least in comparison to the rat. Total doses range from 0.025mg to 1mg, as do the volumes used – normally between 5µl and 10µl(van der Kraan, Vitters et al. 1989, Van Osch, Van Der Kraan et al. 1994, Harvey and Dickenson 2009, Ogbonna, Clark et al. 2012). Before beginning, a preliminary test was completed using 0.025mg, 0.2mg and 0.5mg. Based on the results of these across two mouse strains, the 0.5mg dose was selected as it produced the most reliable and robust change in PWT.

Procedure was carried out as described above for rats, except using 0.5mg monosodium iodoacetate in 5µl of 0.9% saline using a 30G needle. Mice were aged 6-8 weeks, weighing between 20-35g depending on gender and mouse strain.

2.3 Behaviour

2.3.1 Assessment Days

Rats: Animals were assessed on either day 3 (early), day 10 (late) or day 14 (for RVM) post injection, preceding electrophysiology.

Mice: Animals were assessed on day 0, on the morning prior to the injection, and on the mornings of day 3, 7, 14 and 21 there after. If, at any time point, mice displayed obvious pain or physical damage relating to fighting or over-scratching (as appeared in a minority of DTA mice) these animals were excluded.

2.3.2 Acclimatization

Before measurements began, all animals were given a period in which to acclimatize to their new settings – namely a rack of Perspex boxes open at the bottom and top and sat upon a wire grid. This wire grid allowed access to the paws of the animal.

- For the rats, the tops of the Perspex chamber were topped by cardboard to provide a degree of cover and given a period of 20 minutes in which to acclimatize.
- For the mice, black plastic coverings were placed around the sides and as a lid to entirely black out the chambers and the mice left for 1hr – in acknowledgement of their enhanced acclimatization requirements.

During this time, the rooms were off-access to other experimenters to allow a quiet and undisturbed setting.

2.3.3 Mechanical Hypersensitivity

Rat – Punctate Mechanical Hypersensitivity

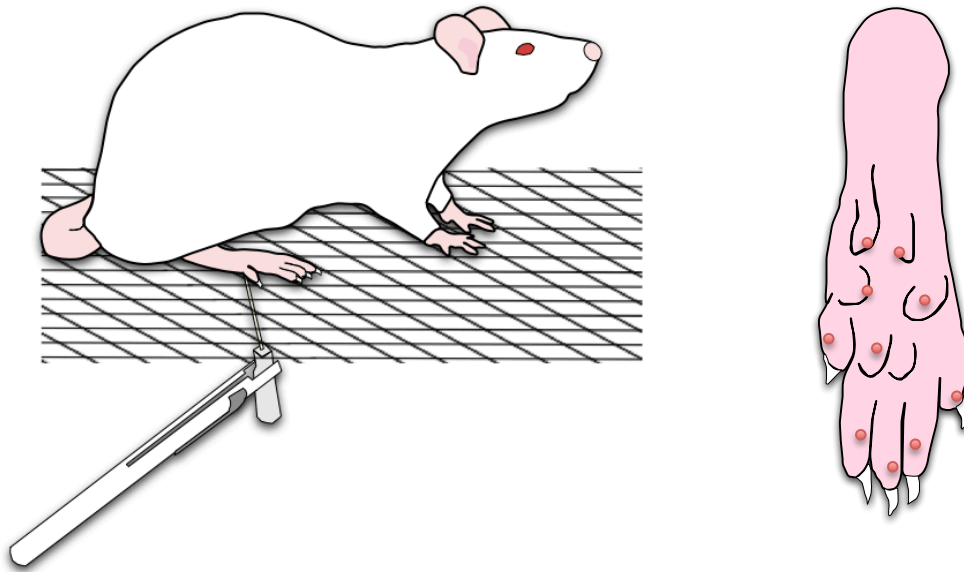


Figure 2.1 – Behavioural testing for punctate mechanical hypersensitivity: Animals were placed in a clear Perspex box, sat atop a metal grid through with hind paws could be reached by vF hairs. vF hairs were applied ten times for each fiber force, once to each of the red spot locations (•) on the hind paw.

Mechanical sensitivity was assessed through the placement of von Frey filaments to the plantar surface of the hind paw and subsequent withdrawal, or not, by the rat. The usually innocuous hairs 1g, 6g and 8g were each applied ten times – once to each toe and once each to the pads on the paw – and the number of withdrawals out of ten recorded. Each hair was applied for 2 seconds, such that the fiber bent, and a withdrawal classified as the active removal of the paw from the stimulus, including full bodily removal from the stimulus; turning to lick the paw; or shaking the paw. Should the fiber slip off the toe before bending this touch was not considered a test, as full force would not have been applied.

These tests were done to both the ipsilateral and contralateral paws in ascending order, such that the 10x1g stimuli would be performed on each paw (contralateral first) and then followed by a one-minute break, cold hypersensitivity test plus additional one minute break before commencing the 6g test.

Rat and Mouse – Paw Withdrawal Threshold

The assessment of change in touch withdrawal threshold was used in both mice and the group of rats to be used in RVM recordings. This technique was adopted, in preference to the one described

above, as it reduced the number of application of the vF hairs while increasing the accuracy of the measurement – to give an value for threshold for withdrawal.

The protocol matches that described above, except that instead of using number of withdrawals out of ten to three separate vF fibers, a paw withdrawal threshold was calculated using the “up-down method”, as described by Chaplan et al(Chaplan, Bach et al. 1994). In brief, vF hairs of sequential increasing or decreasing force are applied, based on the response to the previous stimuli (withdrawal or lack there of). The statistical formula described by Dixon et al is then utilized to calculate the 50% withdrawal threshold(Dixon 1980) – which describes the force at which the animal will withdraw 50% of the time.

Weight-bearing Assessment

The assessment of resting joint discomfort is made using the incapitance test. This was performed in both rats and mice, however a different brand of equipment was used – given the different sizes of the animal. Regardless of animal or machinery, all weight bearing was performed in only those animals deemed calm enough – for example, any rat exhibiting stress induced hiccoughs would be returned to his cage to recover and tested at a later time point.

Rat: Rats were placed in the angled Perspex chamber so that each hind paw rested on a separate force platform, the front paws rested on the Perspex and the tail projected out of the box as seen in Figure 2.2. Animals were given 2 minutes to acclimatize and once stood in the position described above, so that each platform had just one paw in contact, the force exerted by each hind limb was measured over a 5s period to give an average, in grams, for each side. The percentage weight borne of the ipsilateral limb was then calculated. This was repeated 3 times and averaged. (Equipment: Linton Instrumentation, Norfolk, UK)

Mice: Mice were placed on the incapitance tester such that they “hide” their head and front paws in the presented nose cone of their own free will. Some natural degree of exploration of the equipment was allowed to prevent the animal feeling forced into position but gentle tugging of the tail would be used to encourage hiding if necessary. The cone naturally positioned the mouse such that each paw rested on a separate plate, with front paws elevated. The tail was held gently during this time to prevent breaks for freedom or entire submersion into the nose cone tube. Once the mouse was considered to be calm and still, the force exerted by each hind limb was measured over a 10s period, to give an average, in grams, for each side. The percentage weight borne of the IL limb was then calculated. This was repeated 3 times and averaged. (Linton Instrumentation, Norfolk, UK).

Please note that this measure was not performed in rats used for brain recording following the inadvertent disposal of the angled Perspex box required to operate the incapacitance machine. As a result, only PWTs were used to measure behavioural changes in these animals.

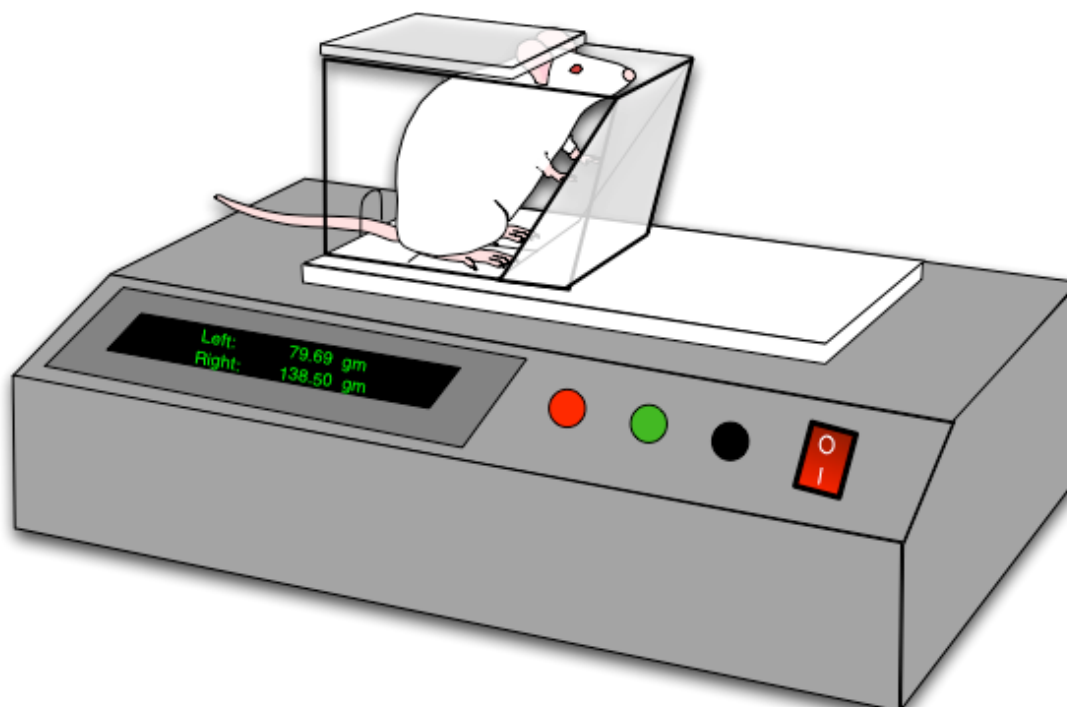


Figure 2.2 - Weight bearing assessment using an incapacitance tester, as describe by Bove et al(Bove 2003). The animal sits in a perspex box that orientates it so that one hindpaw is on each platform, the front paws are elevated and resting on the Perspex, and the tail is projecting out of the box. Once the animal is settled and still a reading is taken which measures the weight borne by each hind paw on each platform.

2.3.4 Cooling Hypersensitivity

Cooling hypersensitivity was measured as the number of withdrawals out of five to the placement of a drop of acetone to the plantar surface of the paw. The drop was placed using a 1ml syringe with plastic tubing attached at the nozzle with which to administer. The tubing was never allowed to touch the animal's paw. Each application was spaced out by at least one minute, including between application to the ipsilateral and contralateral paws.

It should be noted that the syringe was always filled prior to the introduction of the animals to the behaviour room, as the smell of opening the acetone container catches their attention and sometimes unsettles them. This measure was only performed in early and late rats, not in mice or rats to be used for brain recordings, as no change in cooling sensitivity was noted in these earlier experiments.

2.4 Spinal Cord Electrophysiology (Rat only)

This protocol has been previously described (Urch and Dickenson 2003).

2.4.1 Animal Preparation

Animals were placed in a perspex box and weighed. This box was then connected to receive 4% isoflurane in a 2:1 mixture of Oxygen and Nitrous Oxide. Once rats had become unconscious and a-reflexive they were removed from the box and placed on a nose cone, on their backs, and the anaesthetic reduced to 3%. A rectal probe was inserted to provide feedback control of the heating mat, such that body temperature was maintained at 37°C in all animals.

Reflexes were checked before the exposure of the trachea by blunt dissection. A tracheotomy was then performed, involving the insertion of a cannula to the trachea, which was securing with silk thread. Anaesthesia delivery was transferred across to this cannula and reduced to 2.5%. The animals were then fitted into ear bars in a stereotaxic frame, on their ventral side. An incision was then made down the middle of the animal's dorsal side, starting between the shoulder blades and down to approximately the top of the hips – the smaller the incision the better. A mark was then made, through a small scratch, to mark the point at which the ribs met the spine. Above this point, incisions of approximately 2cm in length were made on either side of the spine and a clamp placed here and tightened onto the vertebrae. This served the dual purpose of holding the thoracic region sufficiently high for easy breathing and held the spine securely in place.

At the point of the marker, where the ribs meet the spine, the muscle and connective tissue was removed in a strip of roughly 0.5cm in width and ~0.5cm above and below the marker in order to reveal spinal segments L4-6. A laminectomy was performed. Any meninges still in place were carefully removed with fine (watchmakers) tweezers. Finally, incisions were made (as before) on either side of the spine just below the laminectomy and a clamp tightened onto the vertebrae, such that the spinal cord was held level, straight, tight (cranial-dorsal) and secure. This overall set up formed a natural well around the exposed cord to allow the spinal application of drugs without drip off. Saline was applied when necessary to keep the cord moist throughout the procedure.

Once this set up was complete the anaesthesia was reduced to ~1.6% Isoflurane, such that the animal remained a-reflexive and the breathing rate steady. Throughout the experiment the depth and rate of breathing was monitored visually, along side the colour of the rat ears and tail – the pinkness of which was a good indicator of wellbeing.

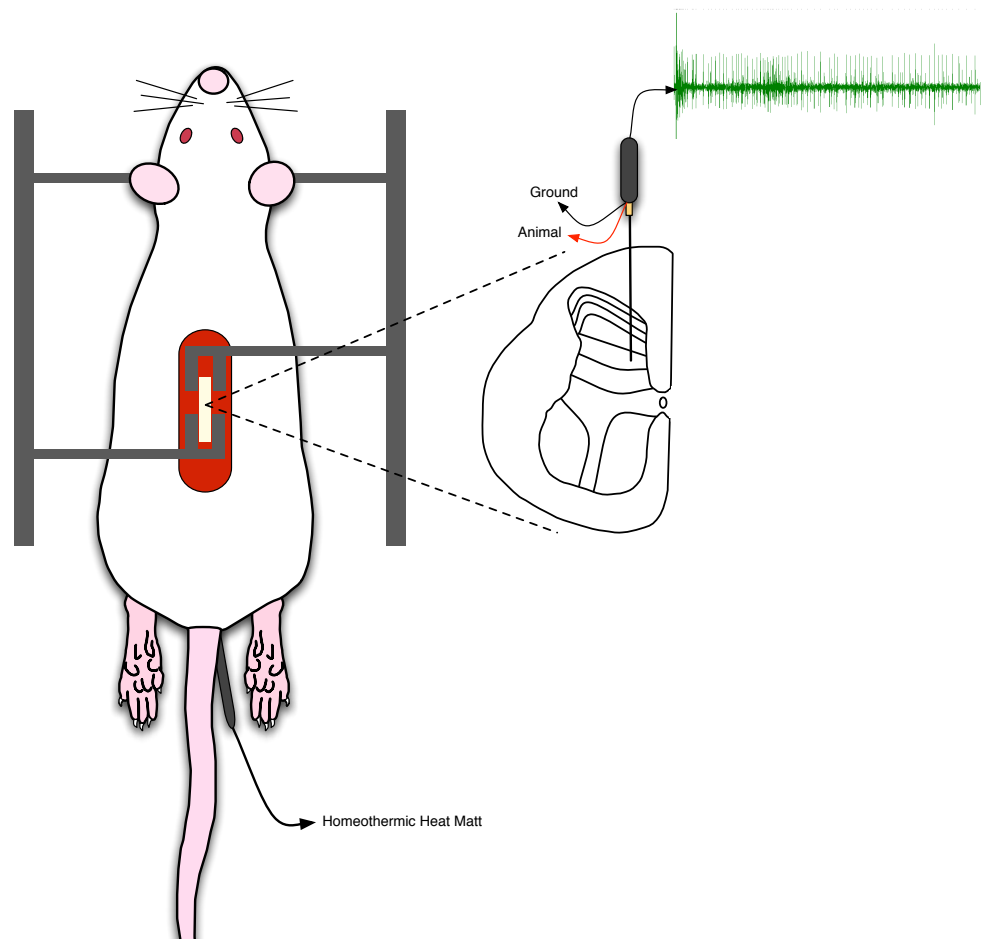


Figure 2.3 - Stereotaxic set up of the rat for spinal electrophysiology – Rat is held in position by a combination of ear bars and 2 sets of clamps above and below the laminectomy. A feedback thermometer sits in the rectum to control the heat mat upon which the rat sits. The electrode is inserted just lateral to the central vessel and lowered to Lamina V. Grounding cables are similarly connected to both the frame and the animal.

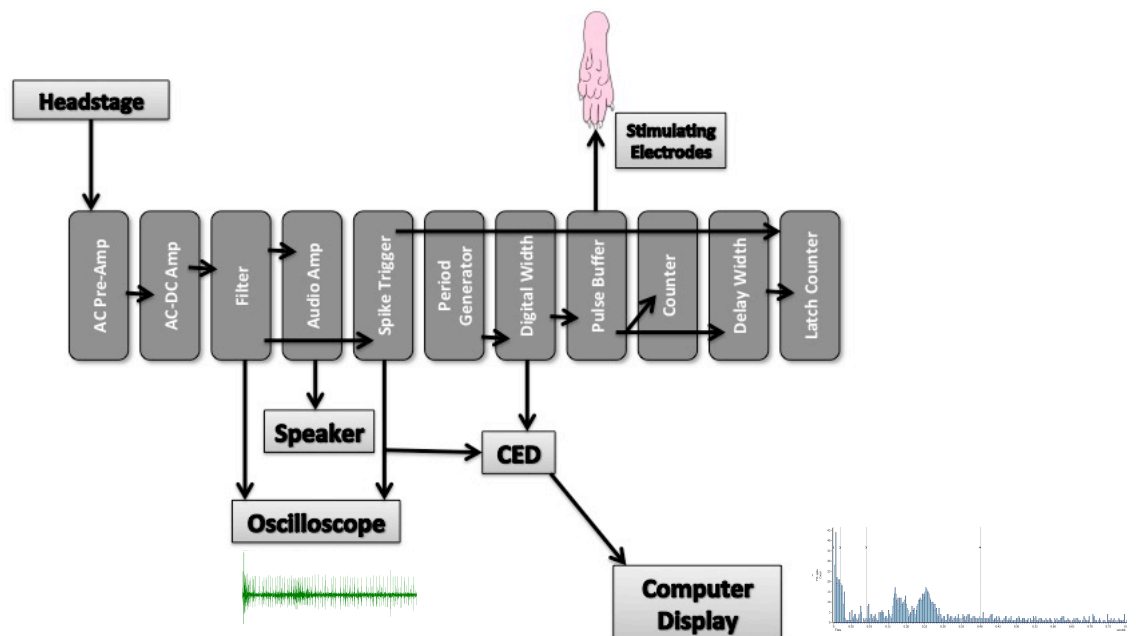


Figure 2.4 - Schematic of the Neurolog recording system, adapted from Urch and Dickenson 2003 (Urch and Dickenson 2003). Input from the spinal cord is transmitted by the electrode through the head stage and into the AC Pre-Amp. The signal is processed such that “noise” is subtracted, the signal amplified and filtered, sending outputs to both the oscilloscope and speaker. Action potentials with an amplitude that exceed the set threshold are outputted to the CED and filtered to the computer. Pre-set frequency electrical stimuli are applied to the paw by the pulse buffer via stimulating electrodes, where all resultant electrical outputs are captured and plotted by the computer into a post-stimulus time histogram which separates activity related to fiber types based on latency.

2.4.2 Single Cell Recordings

Recordings were made by the insertion of a parylene-coated tungsten electrode (AM Systems, Washington, exposed tip 0.1mm, 2M Ω) into the spinal cord of the rat at a depth of approx. 500-1000 μ m – corresponding to Lamina V – using a micromanipulator to visualize the depth. The electrode was held by a head stage which also received connections from two grounding cables – one attached to the animal, to collate electrical activity in the animal not related to spinal cord activity (e.g. heart beat), while the other collected background electrical noise through attachment to the stereotaxic frame. This information was then all fed into the Neurolog AC recording system.

The Neurolog recording system was operated on the A-B setting, signifying differential recording where by signals from the grounding cables is subtracted from those of the electrode to give a cleaner signal. The neurolog system amplifies and filters this signal, feeding it to both an oscilloscope and speakers to allow audiovisual demonstration of the spinal cord outputs. Action potentials were counted by the CED1401 system and fed into a computer for collation, where they were then presented as histograms.

Finding a cell from which to record

The electrode was inserted gently, using a micromanipulator, to the ipsilateral side of the spinal cord such that the electrode would enter just lateral to the central vessel. The electrode was then moved upwards and downwards slowly in the search region (500-1000 μ m from the surface when not puckered) while tapping on the paw. This allowed for the literal sounding out of cells, which were also visualized on the oscilloscope. WDR cells were identified and selected on the following criteria:

- Responding to 8g vF (light touch) and dynamic brush with a minimum of 50 action potentials
- Strong responses to noxious inputs (pinch and 48°C water jet.);
- Responded to natural stimuli in a graded manner, coding increasing intensity.
- Exhibited prolonged firing to a continuing noxious mechanical stimulus, such as pinch.
- A clear signal, which could be recorded without collecting data on neighbours – this was often solvable by fractional movements of the electrode to become more proximal to the cell of interest and further from the neighbour. The signal to noise ratio for stable optimal recording was ~4:1.
- Receptive field which included at least one toe.

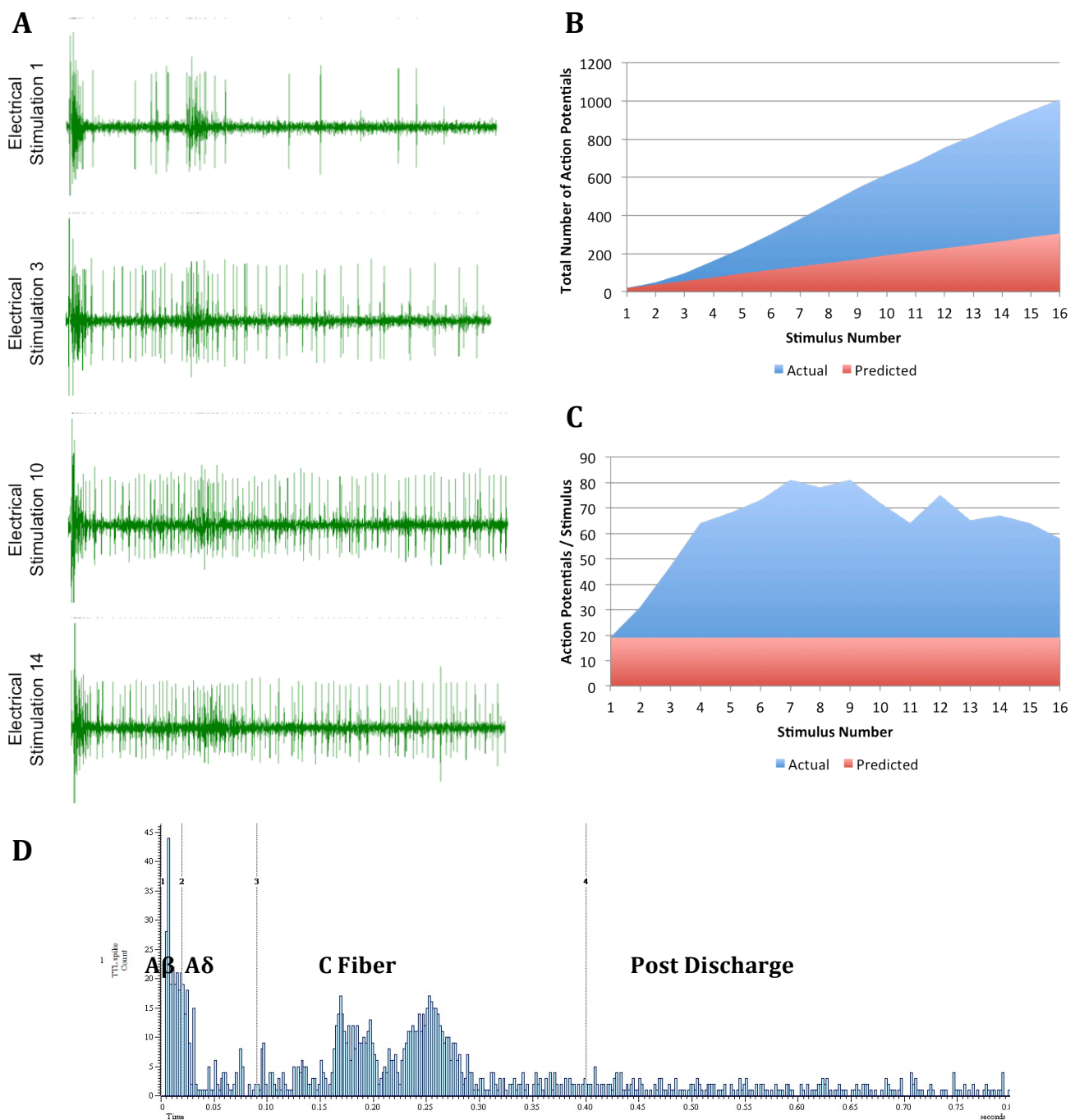


Figure 2.5 – Electrical evoked responses from an example WDR cell. **A)** Evoked responses to sequential electrical stimuli delivered by stimulating electrodes within the WDR receptive field. Each vertical line is an action potential, where each dot above denotes that the computer has “counted” this output. Note the increase in evoked responses between stimulus numbers 1, 3, 10 and 14. **B, C)** Graphical depictions of the actual versus predicted action potential evoked by a train of 16 electrical stimuli at 3 times C fiber threshold for activation. (B = Total generated, C = per stimulus). Shaded areas indicate the basis of the Input (red) and Wind-up (Red) metrics. The first stimulus evoked 19 action potential, the 16th evoked 58. **D)** Histogram separating electrically evoked responses by latency - where action potentials from Aβ arrive much faster than C. Note that the WDR exhibits input from all three fiber classes.

Electrically Evoked Responses

Upon selection of a cell, two stimulating electrodes were inserted into the receptive field of the cell such that they were near but not touching. The threshold for C fiber activation was determined, based on the latency of response to electrical stimuli from these electrodes. A train of 16 stimuli, at three times this threshold stimulating current, were then delivered at 0.5Hz, generating a post stimulus time histogram (PSTH) of electrically evoked responses (**Figure 2.5 D**). Responses were separated into fiber classes, such that each action potential could be attributed to A β , A δ , C fiber of post discharge based on their latency, where post discharges are those responses occurring beyond the C fiber latency period (Urch and Dickenson 2003, Lane, Schnitzer et al. 2010)

During this train of electrical impulses, the total number of action potentials generated so far at each stimulus (as displayed on the neurolog counter) is collected. A value for a predicted no windup response is calculated as the number of responses to the first stimulus multiplied by sixteen, known as the input. This is then subtracted from the final total number of action potentials counted by the final 16th stimulus to give a figure-denoted wind-up. For example – the first stimulus-response is 10 potentials, while the 16th is 1000. Thus the input is $10 \times 16 = 160$. The wind-up is $1000 - 160 = 840$. (See figure 2.5 B and C) This is confirmation of a WDR cell that winds up, completing the final criteria for selection. Note that this is a final selection barrier only performed on cells already deemed WDR – pinning and stimulating before this would create unnecessary tissue damage and consequential peripheral sensitization throughout the (often lengthy) search process.

This electrical stimulation was performed at the beginning of every test run, such that stimuli were delivered in the order [*electrical* \rightarrow *2minute pause* \rightarrow *mechanical* \rightarrow *thermal*]. This pause was normally covered by the time taken recording readings but primarily allowed a brief recovery period for the cell, especially in those showing vigorous after firing. A test run was completed every 20mins until three sets of consecutive, consistent recordings had been collected. This would then be followed by pharmacology (discussed below) and the test runs repeated at specific time points.

Mechanical and Thermally Evoked Responses

Following the 2min pause post electrical stimulation, the natural stimuli are then applied, in order of ascending severity, for 10seconds at one minute intervals to the center of the receptive field – mechanical stimuli preceding thermal. This order and temporal spacing is designed to prevent any sensitization and allow the recovery from any after firing. The stimuli applied, in order, are dynamic brush (ten stroked over the receptive field); 8, 15, 26, 60g vF hairs applied to the center of the

receptive field; 35, 40, 45 and 48°C water jet delivered at a constant force through a 21G needle. The number of action potentials generated over the 10s stimulation period was collected by the computer.

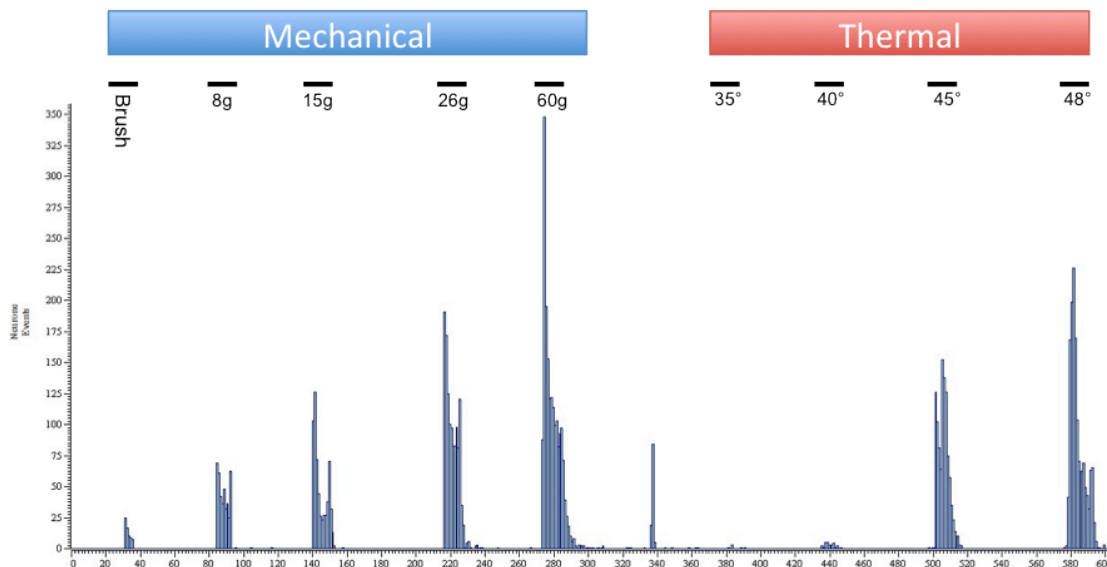


Figure 2.6 – Naturally evoked responses of lamina V WDR neuron. Note the graded response to stimuli of increasing magnitude. The number of action potentials are plotted in 1second bins, where the black bars above denote the 10second stimulation period. These stimuli are brushing of the receptive field (ten strokes – dynamic mechanical stimulation), von Frey hairs and thermal water jets. This cell was selected specifically as it was considered broadly representative, since many cells characterized exhibited limited response to non-noxious thermal stimulation (fewer than 150 action potentials to 35 and 40°C), but respond vigorously at 45 and 48°C.

Once full recordings had been completed the anaesthesia was increased to 5% and death confirmed by cervical dislocation.

2.4.3 Pharmacology

All drugs and controls used during these spinal electrophysiology protocols were applied spinally. This involves the direct application to the surface of the cord. In order to ensure maximum penetrance, the surface of the cord was kept clear of blood clots that could be obstruct access. Once drugs had been applied, test runs were completed at 10, 30 and 50minutes post application. Specifics of the drugs used and their dilution will be discussed in individual chapters.

2.5 Electrophysiological Recordings from the RVM (Rat only)

2.5.1 Animal Preparation

Because the coordinates used to locate the RVM are based on a rat brain atlas for rats between 250-350g, only rats weighed and exceeding 250g could be used in this procedure. Rats found to fall just under this were returned to the animal house for an additional day or two of growth, on a wet mash diet.

Animals were anaesthetized and a tracheotomy performed as previously described. Isoflurane was reduced to 2.5% and rats secured in a stereotaxic frame. An incision was made along the head of approximately 2cm in length and the skull cleared of muscle and connective tissue. Once the skull had “dried out” somewhat, so that bregma and lambda were clearly visible, the incisor bar was adjusted such that these two markers lay equal and the skull was flat. Using a blunt electrode and the micromanipulators, an area corresponding to the center of the RVM was marked on the skull, at 0.5mm mediolateral and 11mm caudal from bregma. This was then slowly drilled, using a dental drill, such that a hole through to the brain of approximately 4mm in diameter was created. The dura was then removed using fine tweezers, if it had not already been by the drilling, and saline dampened gauze placed over the opening to help cease any subsequent bleeding. Anaesthesia was immediately dropped to 1.8% with oxygen alone (i.e. no more nitrous oxide). This was then continuously lowered, in small increments, to 1.3% over the next hour. This was considered a level sufficient to maintain clear reflex withdrawal from paw and tail pinch without spontaneous activity. However, animals were closely and continually monitored throughout for any signs that anesthesia was too light, such as paw withdrawals independent of stimulus, and isoflurane subsequently increased till this was resolved. As previously, depth and rate of breathing was monitored visually, alongside the colour of the rat ears and tail – the pinkness of which was a good indicator of wellbeing – while body temperature was controlled by a heat-blanket, controlled by feedback from a rectal probe.

2.5.2 Single Cell Recording

Recordings were made by the insertion of a parylene-coated tungsten electrode (AM Systems, Washington, exposed tip 0.1mm, 2M Ω) into an area corresponding to the RVM, as mapped by the rat atlas: 0.0-0.9mm mediolateral, 10.5-11.5mm caudal and 9.0-11.0mm dorsal of the dura matter. As described for spinal recordings, the electrode was held by a head stage, which also received connections from two grounding cables, and fed into the Neurolog AC recording system which

filtered, amplified and visualized neuronal activity on an oscilloscope, made audible through speakers, and mapped wave patterns in the Spike 5 software on the computer.

Cells were identified and classified as either ON-, OFF- or neutral cells based on change in firing just prior to tail flick, as induced by 8cm immersion in 50°C water, as described by Fields et al (Fields, Bry et al. 1983). This temperature was selected to prevent sensitization through repetitive immersion. Briefly, cells were characterized as ON cells if they displayed an increased rate of firing just prior to tail flick; OFF by a pause just prior to tail flick; and neutral if they did not change at all. This classification was based upon a change in rate of firing of 7 action potentials per second, and not a percentage as had previously be utilized in this lab simply by virtue of many ON cells having a resting rate of firing of zero, from which a percentage cannot be calculated. This change in rate was calculated by:

$$\text{Rate of Firing due to tail flick} - \text{Mean Baseline Rate}$$

Where the rate of firing due to tail flick was the average rate over a 5 second period composed of the one second before and 4 seconds after tail flick; and Mean baseline rate was the mean rate over a 50 second sample period prior to the application of the noxious stimulus. Some example cells are given in Figure 2.7.

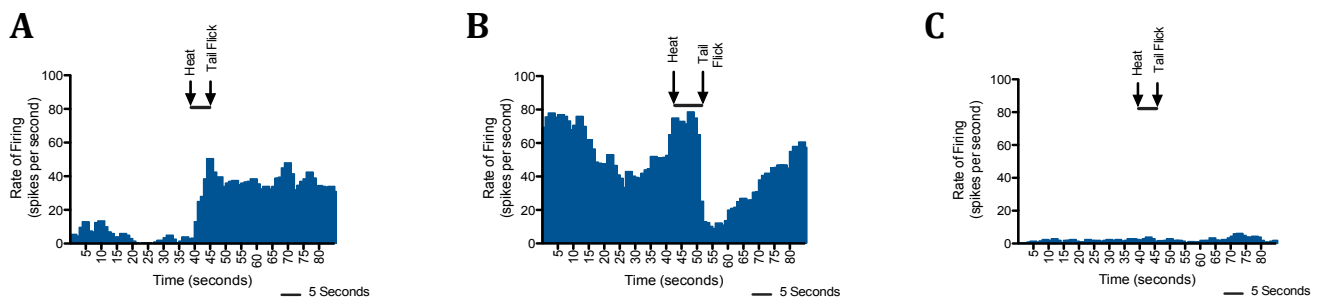


Figure 2.7 - Example ON- (A), OFF- (B) and Neutral (C) cells displaying characteristic change in reflex related firing following noxious thermal stimulation of the tail - Histograms demonstrating the change in rate of firing (spikes per second) of individual ON, OFF and Neutral cells in response to heat evoked tail flick.

While the identification of a single cell, isolated visually on the oscilloscope and audibly through the speakers, was ideal - cells were analysed and followed based upon waveform shape (See 2.5.4). This allowed several cells to be followed in tandem, for example both an ON-cell and neutral cell, unlike in spinal recordings where individual cells were entirely isolated for recording.

2.5.3 Experimental Protocol

Once a cell was identified and classified, it was characterized through three phases of testing:

1. Change in rate of firing in response to tail flick.
2. Mean resting activity over 15minutes.
3. Change in rate of firing in response to ipsilateral and contralateral stimulation of the paw and knee.

Change in tail flick reflex related firing

Once a cell was identified, the tail flick reflex and changes in related RVM firing were characterized. This involved three rounds of tail flicks, spaced 5mins apart. All time point markings were made manually onto the spike system, based on experimenter observation: H notified the point at which the tail was immersed, to 8cm, in 50°C water. T notified the point at which tail flick could be observed to have begun. The time elapsed between H and T was denoted the latency to tail flick, and is given as the mean of these three tail flicks. Change in rate of firing was calculated, as described above, and the greatest change in rate of the three trials used for analysis.

Mean resting activity

The rat was allowed 5mins following the third tail flick. Resting activity was then recorded as the rate of firing (spikes per second) in 1minute segments and averaged to give a mean rate, termed baseline firing.

Knee and Paw Stimulation

The mechanical stimuli were applied, in ascending order of force, first to the ipsilateral paw and knee, and then to the contralateral side. The von Frey fibers were applied at one minute intervals to the middle toe such that the fiber was in contact and bent for 20 seconds. If observable changed in firing continued beyond the one-minute interval, additional time was left for the cell to recover to baseline rate of firing. Fibers used were: 8, 15, 26, 60 and 100g. Following these, the knee joint was

clamped for 20seconds by calibrated forceps set to close to a point smaller than the knee diameter. This protocol was repeated three times, with 10minute pauses between each round of stimulation.

Changes in rate of firing were calculated as:

$$\text{Rate of Firing During/After stimulus} - \text{Mean Baseline Rate}$$

Where baseline was calculated as the mean rate of firing for the 20 seconds directly preceding the stimulus; Rate of firing during was calculated as the mean rate of firing for the 20seconds during von Frey or clamp application; and the rate of firing after stimulus was the mean rate in the 20seconds after the stimulus. The greatest change in rate of firing over the three rounds was then used for analysis. This measure of after stimulus change in rate of firing was calculated following observations that the increase or pause in firing sometimes followed the release of the more noxious stimuli, namely the clamp and 60 and 100g von Frey hairs.

2.5.4 Data extraction

During these experiments Spike 2 recorded all raw data as individual waveforms. Once the experiment was complete, Spike2 was used to analyse these waveforms into individual wavemark channels. These channels use the shape of the waveform (including amplitude and duration) to form templates, where any spike that fits within this template is classed as originating from the same cell. In some instances, two templates are created that may look like the same cell based on the template shape, identical activity of the two cells and the waveform heights. In these instances, the two separate templates were compared using a principal components analysis and if considered to be the same, through mostly overlapping components, these templates were merged into one. Each individual wave mark was then converted to rate (spikes/second) and data extracted.

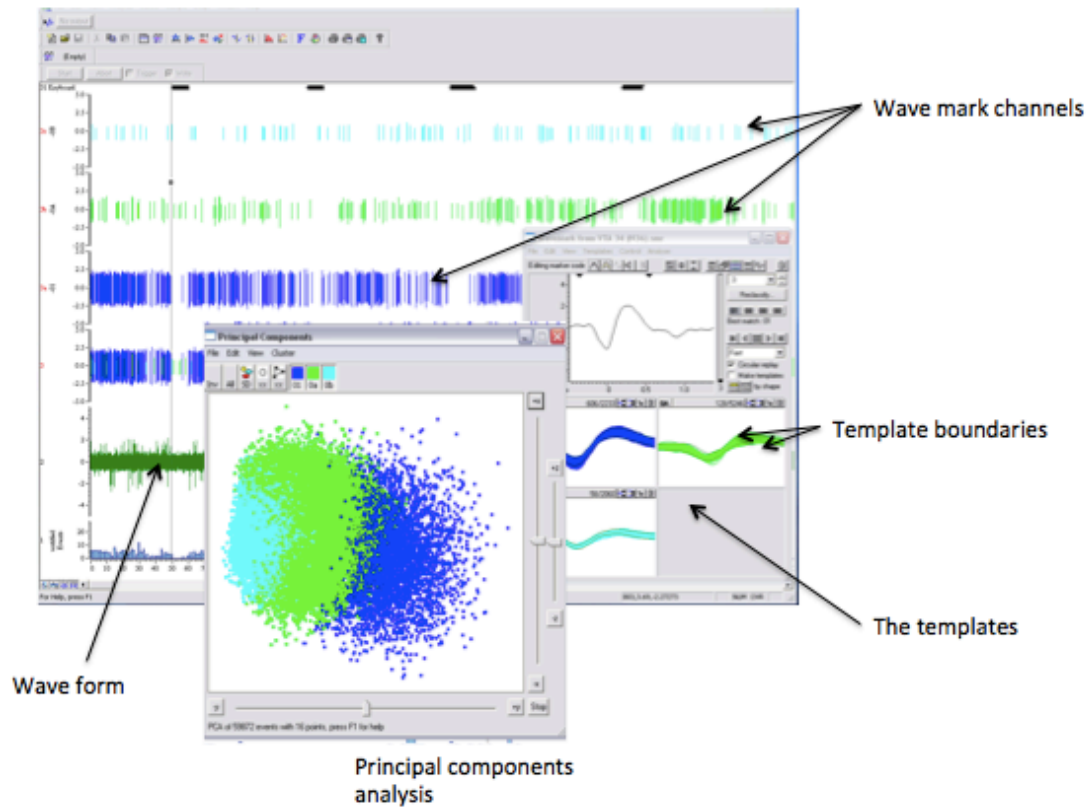


Figure 2.8 – Depiction of the process through which Spike 2 waveform data is sorted and filtered to identify individual cells for extraction and analysis: The original waveform is analysed to produce wavemark templates that allow spikes to be attributed to individual cells. These templates are based on the shape of the action potentials detected, which vary both because of the individual cell ion channel populations and proximity. The similarity of these templates can then be assessed by principal components analysis to ensure all templates represent different individual cells.

2.6 Analysis

All graphs were drawn up using Graph Pad Prism 5.0, as were all statistical analyses. Please refer to individual Chapter methods for the individual stats protocols used.

Note:

- In behavioural studies, data is either expressed as mean difference (e.g. Ipsi Withdrawals out of ten – Contra Withdrawals out of ten), the mean percentage incapacitance or as PWT, as calculated by Dixons non parametric statistical analysis.
- Where baseline is referred to in spinal electrophysiology data, these are the measurements taken prior to drug administration and represent the mean of three stable test runs. The effect of each drug is expressed as the maximum change from this baseline value per individual drug dose across all time points. Graphs are expressed as raw action potentials \pm SEM.
- Where baseline is referred to in RVM recordings, this is the rate of firing prior to the application of somatic stimuli. For both tail flick, paw and knee stimulation related changes in rate of firing, the value given is the maximum change from baseline value. Graphs are expressed as either rate of firing (spikes per second) or change of rate in firing.

Chapter 3 – Baseline Profile of 1mg MIA Rats

As discussed in section 1.5 of the introduction, there remain inconsistencies in the literature around the exact expected profile of evoked spinal cord electrophysiology in this selected model of OA pain, the MIA model. As I am using a lower dose of MIA than has traditionally been used to model OA pain in spinal electrophysiological investigations in this lab, it seemed doubly important to clearly characterize the baseline behaviour and evoked spinal cord electrophysiology of the rats used to investigate changes in descending controls in OA. As such, this chapter is a precursor to the pharmacology work in Chapter 4, in which the baseline readings (pre-pharmacology recordings) of all animals used in Chapter 4 have been merged to provide an overview of the baseline profile of these animals. The baseline readings have been merged into one of four experimental cohorts – Early MIA, Late MIA, Early Sham and Late Sham – in the hope of providing greater space to focus on the pharmacology outcomes in Chapter 4.

3.1.1 The importance of dose

As discussed in section 1.5, it is well established that the MIA model has a dose dependent effect in the induction of both histopathology and pain like behaviour in animals (Guingamp, Gegout-Pottie et al. 1997, Janusz, Hookfin et al. 2001, Thakur 2012, Udo, Muneta et al. 2016). While this is logical, since a larger dose will provoke the more rapid and wide spread changes, it also raises questions about the basis for the pain we are modeling and whether this is representative of patient groups.

Within this thesis a dose of 1mg of MIA has been selected to investigate adaptations to descending controls, and later to RVM activity in rats, the results of which are presented in Chapter 4 and 5 respectively. The reasoning behind the selection of this dose is three fold:

1. A 1mg dose of MIA has been shown in past literature to produce a robust, reliable, OA-like histopathological and pain profile
2. Previous literature has characterized a neuropathic profile in the higher, 2mg dose of MIA
3. Previous literature characterizing changes in descending controls in the MIA model have used this higher, 2mg model of OA pain

In considering these factors, discussed further below, it became clear that there could be important differences between the changes in descending controls observed in the 1mg model of MIA which had yet to be characterized.

3.1.1.1 The 1mg MIA model: a reliable and robust model of OA pain and pathophysiology

While a consensus may not exist for the expected adaptations observed to lamina V WDR cell evoked activity in the 1mg MIA model of OA, there is a much greater degree of consensus around the time course and extent of structural changes in this model - changes which also show a relationship with the dose administered in both the time course and severity of changes (Guingamp, Gegout-Pottie et al. 1997, Janusz, Hookfin et al. 2001, Thakur 2012, Udo, Muneta et al. 2016).

The choice to pursue the 1mg dose flowed out of recent work conducted by M. Thakur in his 2012 thesis, which in part looked to characterize the physiological and structural differences between a 1mg and 2mg MIA model (Thakur, Rahman et al. 2012). This work characterized a neuropathic component in the 2mg but not 1mg model (see 3.1.1.2 below), with measures including assessments of histology and proteoglycan loss (Figure 3.1), DRG immunohistochemistry, quantification of total DRG ATF-3 expression, quantification of intra-epidermal nerve fibre density in plantar hindpaw and quantification of microglial activation, along side the additional behavioural, electrophysiological and pharmacological measures. From this work there was a clear conclusion that a 1mg dose had a robust OA like profile, with significant proteoglycan loss in both the 1mg and 2mg MIA models, in addition to the development of a robust OA pain like profile (Thakur, Rahman et al. 2012).

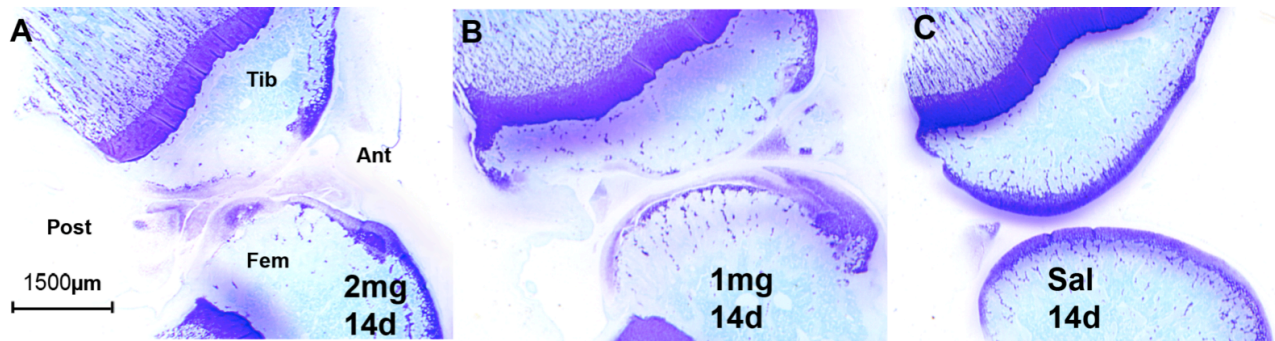


Figure 3.4 – Articular histology taken from Thakur et al 2012, comparing the relative effect of saline, 1mg or 2mg MIA on articular cartilage at 14 days after injection. Sagittal sections are stained with toluidine blue to visualize cartilage proteoglycan content. Fem= femoral condyl, Tib = tibial condyl. Ant = anterior aspect of knee, Post = posterior aspect. A) 2mg B) 1mg C) Saline (Thakur, Rahman et al. 2012)

However, numerous other 1mg MIA studies have characterized further components of the 1mg MIA model at the time points investigated, including:

- The development of an initial inflammation (Bove 2003, Guzman, Evans et al. 2003, Clements, Ball et al. 2009, Mapp, Sagar et al. 2013)

- Proteoglycan loss and chondrocyte death (Guingamp, Gegout-Pottie et al. 1997, Gencosmanoglu, Eryavuz et al. 2001, Pomonis, Boulet et al. 2005, Ivanavicius, Ball et al. 2007, Sagar, Staniaszek et al. 2010, Strassle, Mark et al. 2010, Stevenson, Mercer et al. 2011, Thakur, Rahman et al. 2012) (Bove 2003, Guzman, Evans et al. 2003, Clements, Ball et al. 2009, Mapp, Sagar et al. 2013)
- Cartilage fibrillation (Guingamp, Gegout-Pottie et al. 1997, Gencosmanoglu, Eryavuz et al. 2001, Guzman, Evans et al. 2003, Ivanavicius, Ball et al. 2007)
- Subchondral bone sclerosis and osteophytes (Pomonis, Boulet et al. 2005, Ivanavicius, Ball et al. 2007, Clements, Ball et al. 2009, Nagase, Kumakura et al. 2012) (Guingamp, Gegout-Pottie et al. 1997, Gencosmanoglu, Eryavuz et al. 2001, Guzman, Evans et al. 2003, Ivanavicius, Ball et al. 2007, Mapp, Sagar et al. 2013)
- Development of pain like behaviour, including shifts in WB, reduced PWT, gait adjustments and lost of spontaneous activities (Guingamp, Gegout-Pottie et al. 1997, Guzman, Evans et al. 2003, Ivanavicius, Ball et al. 2007, Clements, Ball et al. 2009, Sagar, Staniaszek et al. 2010, Strassle, Mark et al. 2010, Stevenson, Mercer et al. 2011, Nagase, Kumakura et al. 2012, Thakur, Rahman et al. 2012)

Some of these changes and their time-course are visualized in Figure 3.2, taken from Ivanavicius et al 2007. Further detail and a discussion of the time course can also be found in section 1.4.

The characterization and publication of such outcomes in a 1mg model of MIA consequently gives us confidence that the 1mg MIA model is just as robust and reliable a model of OA pain as the 2mg MIA model, where the joint histopathology and pain characteristics appear broadly translatable to OA in patients.

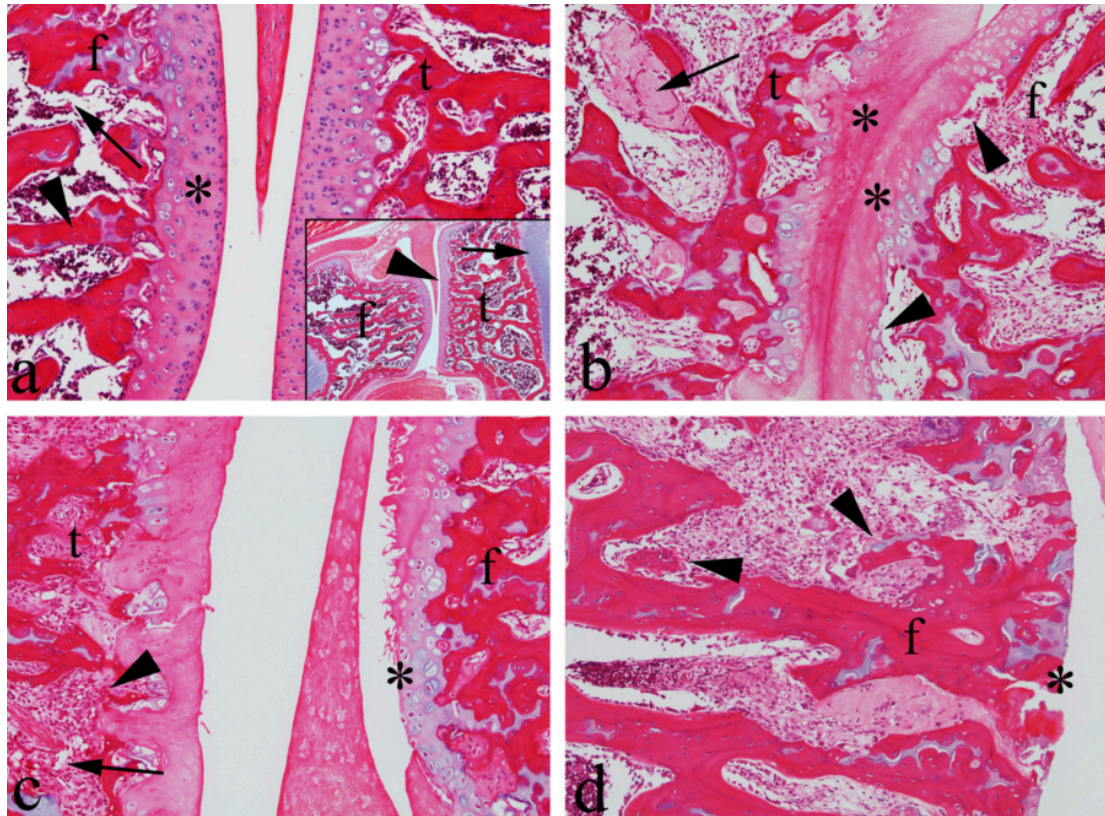


Figure 3.2 – Articular histology taken from Ivanavicius et al 2007 displaying the time course of the histopathology of a 1mg MIA injection, (f, femoral condyle; t, tibial condyle). a) saline control knee at 8 days, where the * highlights normal, healthy cartilage overlying the subchondral bone plate and cancellous bone of the epiphysis (arrowhead) which is interspaced by marrow cavity (arrow) b) MIA injected knee at 8 days, highlighting the pallor of the intact articular cartilage and ghosting of chondrocytes, with some degeneration of the underlying subchondral bone (arrowheads). The arrow highlights where fibrous tissue is developing in the marrow cavity c) MIA injected knee at 12 days, where * highlights erosion of the articular cartilage with marked subchondral bone degeneration and loss (arrowhead) d) MIA injected knee 14 days – pronounced ulceration of the articular cartilage (*) and superficial subchondral bone with ongoing degeneration and remodeling of deeper cancellous epiphyseal bone (arrowheads). (Ivanavicius, Ball et al. 2007)

3.1.1.2 Neuropathic profile of 2mg MIA rats

The development of a neuropathic-like pain component in the MIA model has been identified recently(Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012), though some debate remains as to whether this is a flaw of the model as opposed to effective characterization of OA pain pathology. As mentioned, recent work from this lab has indicated that larger concentrations can induce neuropathic like changes(Thakur, Rahman et al. 2012). In this study, in which 1mg and 2mg MIA rat groups were compared, it was found that the 2mg animals showed a significant reduction in fiber density in the plantar hind paw skin, a significant increase in ATF-3 expression and microgliosis in the spinal cord, which was not observed in the 1mg animals, indicating that the larger dose could induce significant axonal injury(Thakur, Rahman et al. 2012). These suggestions of mild neuropathy in the MIA model have been made before in previous studies(Ivanavicius, Ball et al. 2007, Orita, Ishikawa et al. 2011).

It seems likely that all MIA animals will eventually develop a neuropathic pain state, as pathology advances and lesions affect nerve endings in the bone. However, dose is pivotal to when this switch from a nociceptive and inflammatory pain to a neuropathy driven pain occurs. This work by Thakur et al would indicate that in the 2mg model this switch to neuropathy is already underway at 14days, while the 1mg model is predominantly nociceptive pain(Thakur, Rahman et al. 2012).

The important implication is that the dose and time points investigated may define the relevance of findings from animal models. Consider the efficacy of pregabalin to attenuate behaviour and reduce the WDR evoked neuronal responses in animals treated with 2 mg MIA, but not 1 mg or sham treated groups(Rahman, Bauer et al. 2009, Vonsy, Ghandehari et al. 2009, Thakur, Rahman et al. 2012). This suggests that gabapentinoids are effective pain relief in OA pain with neuropathic contributions, but not in all patients. This would explain why these results have not translated to clinical use of these drugs in OA management, since the majority (between 68-80%) of patients' OA pain is non neuropathic(Hochman, Davis et al. 2013, Oteo - Álvaro, Ruiz - Ibán et al. 2014). This would similarly explain the progressive loss of efficacy of celecoxib or diclofenac, but not opiates, during more advanced time points in the higher doses of the MIA model(Fernihough, Gentry et al. 2004, Pomonis, Boulet et al. 2005).

The presence of potential neuropathy in the higher dose models is not necessarily a limitation of the MIA model, since patients with neuropathic pain relating to their OA are becoming increasingly well characterised. OA patients use similar descriptors for their pain to neuropathy patients (Hawker, Stewart et al. 2008, Shigemura, Ohtori et al. 2011), such as burning, electric shock or numbness, and on questionnaires like PainDETECT and *Douleur Neuropathique in 4 questions (DN4)* patient sub groups are identified as having neuropathic-like pain(Gwilym, Keltner et al. 2009, Oteo - Álvaro, Ruiz - Ibán et al. 2014). The use of QST to document symptoms such as loss of sensations, notably to proprioception and vibration sensitivity at cutaneous sites, has provided further evidence of a neuropathic like component(Hurley, Scott et al. 1997, Sharma 1999, Shakoore, Agrawal et al. 2008, Felson, Gross et al. 2009, Hochman, Davis et al. 2013). In light of this, the PainDETECT survey has been modified (mPD-Q) to try to more accurately identify this cohort with a view to providing better analgesia(Hochman, Gagliese et al. 2011). The combined uses of mPD-Q and QST point to a patient population of ~20% with neuropathic contributions to their pain(Hochman, Davis et al. 2013), though more recent work has suggested this could be as high as 52% (or 33% once potential confounders were excluded)(Oteo - Álvaro, Ruiz - Ibán et al. 2014).

As such, the OA patient population is clearly composed of sub groups whose pain can be differentially attributed to mixtures of nociception, inflammation and sometimes neuropathy. Identifying where patients fit may enable clinicians to identify more suitable and effective analgesics e.g. pregabalin, Tricyclics, duloxetine etc. (Hochman, Davis et al. 2013), but similarly knowing where on this sliding scale of OA pain mechanisms each dose and time point of the MIA model represents may produce more clinically informative conclusion. As such there appears value in investigating the mechanisms contributing to pain in OA in both the 1mg and the 2mg model.

3.1.1.3 Previous literature has characterized changes in descending controls in a 2mg MIA model of OA pain

As is discussed in much greater detail in the introduction and discussion sections of Chapter 4, previous work in this laboratory and others has sought to characterize the potential adaptations of descending serotonergic and noradrenergic controls in the 2mg MIA model of pain (see Section 4.1.1 and 4.1.2). Specifically, this work identified time-dependent changes in the noradrenergic and serotonergic descending controls mediating spinal excitability, and thus pain perception, in a 2mg model of OA pain compared to sham and naïve controls (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). As we have discussed above, this dose of MIA has been suggested to be driving neuropathy (Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012). This then raises questions about the potential differences in modulation between a neuropathic and non-neuropathic MIA rat, given the extent of adaptation of descending controls has been shown to be variable between different pain conditions (Green, Scarth et al. 2000, Rahman, Suzuki et al. 2004). It is hypothesized that, at this lower, non-neuropathic dose of MIA, modifications in the descending control on spinal excitability by NA and 5HT, as revealed by the spinal application of various antagonists, may also differ. In other words, the conclusions drawn previously regarding the descending control of the 2mg model of pain may only be applicable and translatable to a neuropathic sub population of OA patients – but this will only become more apparent once any changes have been characterized in a non-neuropathic MIA OA pain rat.

3.1.2 Chapter Aims

In order to understand better the differences in pain mechanisms that may operate between a 1mg and 2mg MIA model of OA, this thesis has used a 1mg dose of MIA in all rat work. I took the view that a 1mg model could be considered a model of milder OA, representing non-neuropathic earlier stage OA patients, and sought to confirm similarities or highlight differences in descending modulation of OA pain which had previously be defined by this lab in the 2mg model (See Chapter 4).

However, the pain profile of the 1mg MIA model is not as well established, though mechanical sensitivities at the hind paw withdrawal have been documented(Sagar, Staniaszek et al. 2010, Thakur, Rahman et al. 2012, Sagar, Nwosu et al. 2015). When additionally taking into account the inconsistencies in the electrophysiological profiles in published literature (See Section 1.4) it appeared valuable to clearly characterize the baseline behavioural and electrophysiological profile of these 1mg MIA rats.

The aim of this chapter is to present the collective baselines of experimental work discussed in Chapter 4, with a view to evaluating the overall behavioural and electrophysiological changes observed at two stages of a lower dose, 1mg MIA model: The early phase – where pain is characteristically driven more by inflammation and peripheral effects of MIA injections; The late phase – where pain is characteristically driven more by chronic nociception and central sensitization, but crucially not neuropathy in this dose, at this time. This would then provide more space for the evaluation of pharmacological outcomes in Chapter 4.

3.2 Methods

3.2.1 Animals

All work was conducted in Male Sprague Dawley rats, bred and housed in the Central Biological Services Unit at University College London. As described in Chapter 2, behaviour and electrophysiology was conducted either at the early stages of OA pain development, at days 3-5 after injection, or late stages, at days 10-14 after injection in animals in the range of 220-250g, such that they were injected at either:

- **Early:** MIA (OA) or Saline (sham) injection into animals weighing 160-180g.
- **Late:** MIA (OA) or Saline (sham) injection in animals weighing 120-140g.

As such, four populations of experimental animals were: Early MIA, Early Sham, Late MIA and Late Sham.

3.2.2 Induction of the model

As detailed in Section 2.2, rats were injected with 1mg MIA (Sigma, UK) to the left knee.

3.2.3 Behavioural Assessment

Rats were assessed at either Day 3 (Early) or Day 10 (Late) following injection to assess punctate mechanical hypersensitivity, cooling hypersensitivity and incapacitance. The protocol sequence was as follows:

Acetone → vF 1g → Acetone → vF 6g → Acetone → vF 8g → Acetone → Acetone → Incapacitance

All tests were performed at one-minute intervals such that contralateral and ipsilateral paws in tandem, where the contralateral paws were always tested first, with a one-minute interval before the ipsilateral test. In other words: *Acetone (CL) → Acetone (IL) → vF 1g (CL)* and so on. Details of the exact protocol and withdrawal criteria are given in Section 2.3.3 and 2.3.4.

3.2.4 Electrophysiological Assessment

Spinal electrophysiology was carried out in rats weighing 220-250g at either days 3-5 (Early) or days 10-14 (late) after injection of either MIA or Saline, as described in detail in Section 2.4. The results presented in this chapter are the collated baseline recordings of all WDR spinal electrophysiology in MIA or sham rats, both early and late, used in this thesis, where the baseline is the mean of three rounds of 20minute recordings of electrical, mechanical and thermally evoked stimuli.

3.2.5 Data Analysis

All data is presented as the mean \pm SEM.

Behaviour

- Punctate mechanical and cooling hypersensitivity: Data expressed as the mean difference in the number of withdrawals out of ten. Difference between groups analysed using a non-parametric Mann-Whitney U test.
- Incapacitance: Data expressed as the percentage of total weight borne on the ipsilateral side. Analysed using an un-paired student's T test.

Electrophysiology

- Naturally evoked (mechanical and thermal) responses: Data expressed as number of action potentials. Difference between groups analysed using a two-way ANOVA followed by Bonferroni post-hoc tests.
- Electrically evoked and dynamic brush responses: Data expressed as number of action potentials. Difference between groups analysed using unpaired student's T test.

Values were deemed significant at $p < 0.05$.

3.3 Result

3.3.1 Behavioural hypersensitivity in the MIA model of OA

Punctate mechanical and cooling hypersensitivity and incapacitance was assessed in Rats following the intra-articular injection of MIA and compared to sham controls, which received saline. These tests revealed the development of a significant ipsilateral punctate mechanical hypersensitivity and reduction in percentage weight borne in MIA animals versus shams in both early and late groups.

While no difference was observed to vF1g, vF6g or acetone between MIA and Sham in either time group, at vF8g MIA animals from both early and late groups showed a significantly greater propensity to withdraw the IL paw than sham equivalents (Figure 3.3 A and B; n=13-15; Mann Whitney-U; ** $p \leq 0.01$; * $p \leq 0.05$).

Similarly, MIA animals exhibited a reduction in the percentage total weight borne on the ipsilateral paw compared to time matched shams, such that in the early animals: $43.2 \pm 1.9\%$ vs. $51.8 \pm 1.7\%$ MIA vs. Sham; and in late animals: $42.7 \pm 2.7\%$ vs. $53.1 \pm 1.5\%$ MIA vs. Sham (Figure 3.3 E; Unpaired Students T-test; ** $p \leq 0.01$).

No differences in withdrawals to punctate stimuli, acetone or incapacitance were observed between either Early vs. Late MIA animals or Early vs. Late Sham animals (Figure 3.3 C, D and E; n=13-15).

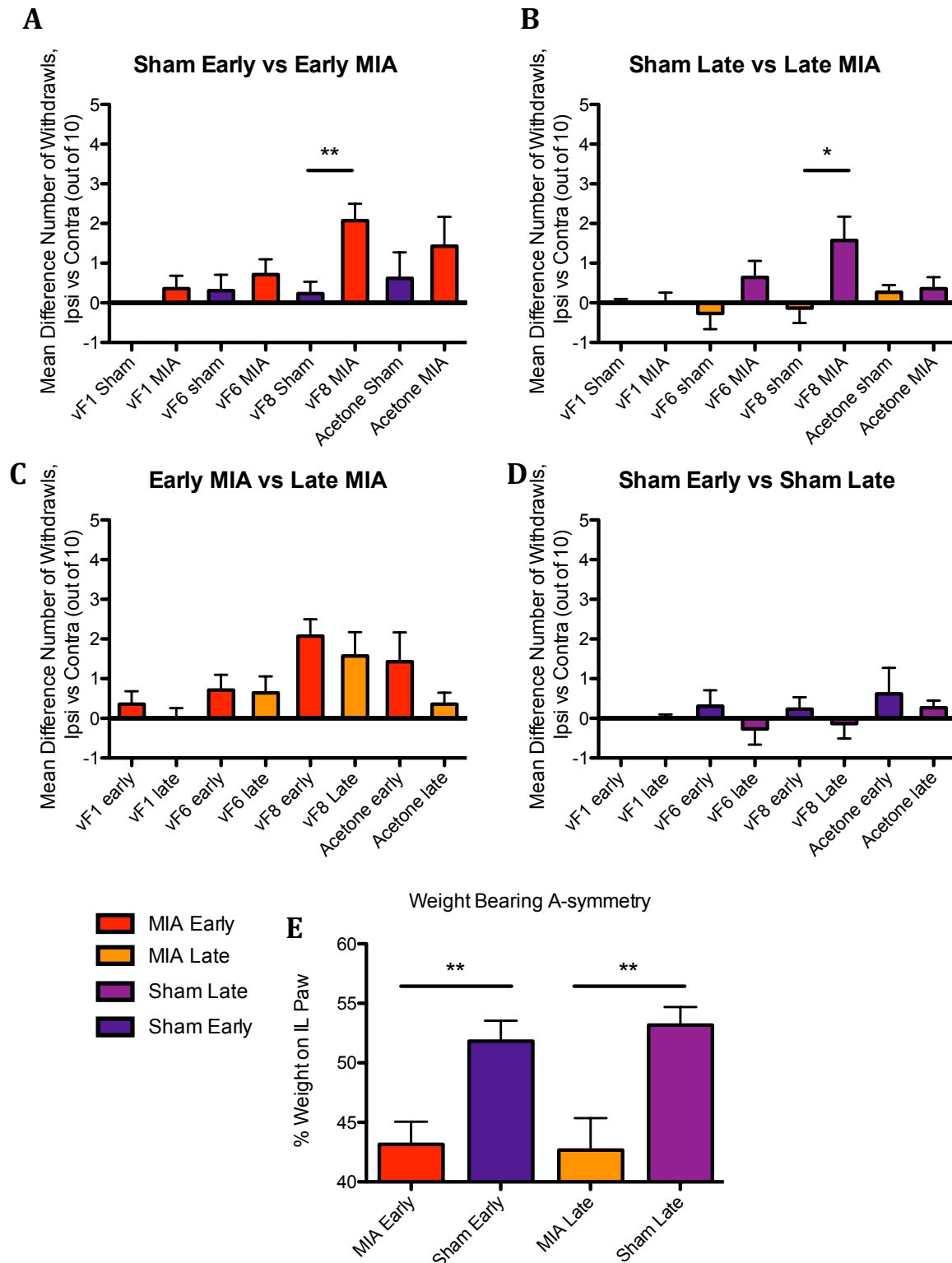


Figure 3.3 – Monosodium Iodoacetate induced OA produced significant punctate mechanical hypersensitivity and a reduction in ipsilateral weight bearing versus shams in both early and late stages: A) At day 3 following injection, MIA animals show significant hypersensitivity to vF8g compared to sham animals (Sham n=13, MIA n=14). This effect is replicated at day 10 following injection (**B**) (Sham n=15, MIA n=14). **C)** Arthritic animals receiving 1mg MIA injection show no difference at days 3 or to animals at day 10 in withdrawal from punctate stimuli. (Early MIA n=14, Late MIA n=14). **D)** Sham animals receiving saline injection show no difference across all behavioural measures at days 3 or 10. **E)** At both day 3 and 10 following MIA injection a reduction in ipsilateral weight bearing versus sham is observed, with no different between either early MIA early and late, or Sham early and late animals.

3.3.2 Spinal Neuronal Hypersensitivity in the MIA model of OA

Evoked responses of WDR cells following electrical, mechanical and thermal stimuli of the IL hind paw were assessed in MIA and Sham animals at either the early or later stages. These tests revealed enhanced responses of Early MIA animals to mechanical, both dynamic brush and punctate, and thermal stimulation in comparison to Early Sham animals.

Electrically Evoked Responses

Across all groups of animals, no significant effect was observed on the electrically evoked responses of Lamina V WDR cells (Figure 3.4A). There is a trend towards a greater C fiber count and input in Early MIA animals compared to Early Sham animals, but this trend is not mirrored in Late MIA and Sham animals, as there is similarly a trend towards greater C fiber count and input in Late Sham compared to Early Sham.

Dynamic Brush Evoked Responses

Early MIA animals exhibited a significant increased in the number of action potentials evoked in response to a dynamic brush stimulus to the receptive field in comparison to Early Sham (Figure 3.4B. Un-paired Student's T test; * $p \leq 0.05$). While no difference is observed between Late MIA and Sham groups, the difference between Early MIA and Late MIA nears significance at $p=0.058$.

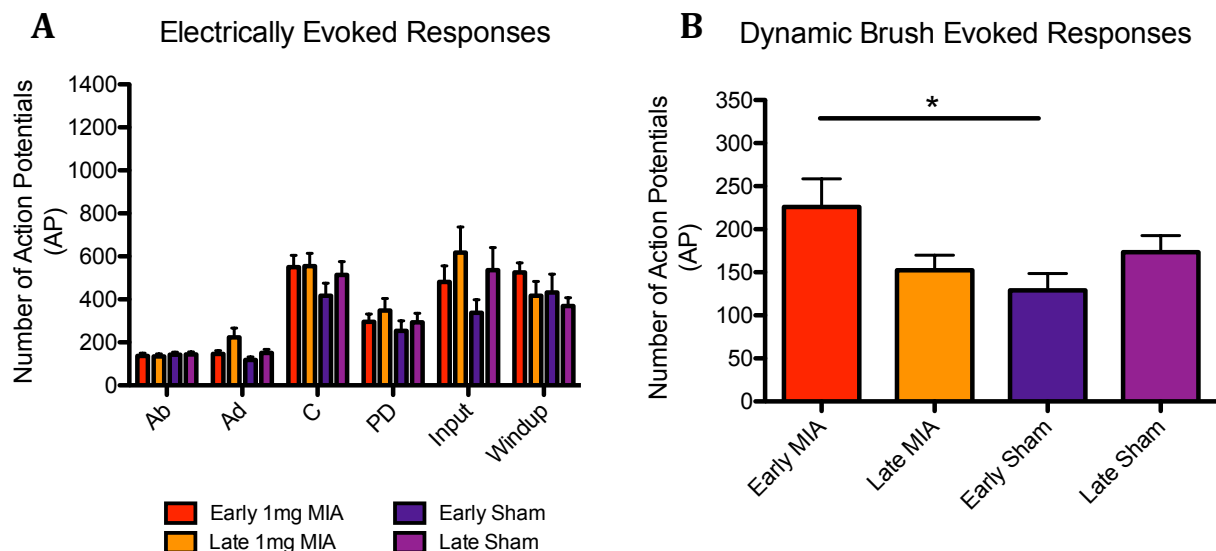


Figure 3.4- Comparison of electrically and dynamic brush evoked responses of Lamina V WDR cells in MIA and Sham animals at either Early or Late stages after injection. A) No difference in the electrically evoked responses of WDR cells is observed in either the Early or Late MIA animals versus shams. **B)** Dynamic brush evoked responses are significantly enhanced in Early MIA animals versus Early Sham (* $p \leq 0.05$) ($n=13-15$).

Punctate Stimulation Evoked Responses

The evoked responses of Lamina V WDR cells following the application of vF hairs to the receptive field in the IL paw produced a stimulus-response curve, whereby the increasing force evoked a greater number of action potentials. Two-way ANOVA found that this stimulus response relationship was significantly enhanced in Early MIA animals compared to their Sham counterparts (Figure 3.5A: $p=0.015$), where Bonferroni post hoc tests indicated that these evoked action potentials were significantly greater in response to noxious punctate stimulation. The number of action potentials evoked by vF26g and vF60g was significantly greater in these Early MIA animals compared to early Sham (Figure 3.5A: ** $p\leq 0.01$; * $p\leq 0.05$). This trend was observed to lower force punctate stimulation, where both vF8g and vF15g near significance ($p=0.077$; $p=0.052$ respectively).

No difference was observed in the number of action potentials evoked in Late MIA and Late Sham animals (Figure 3.5B). Similarly no difference was observed between Early and Late MIA animals,

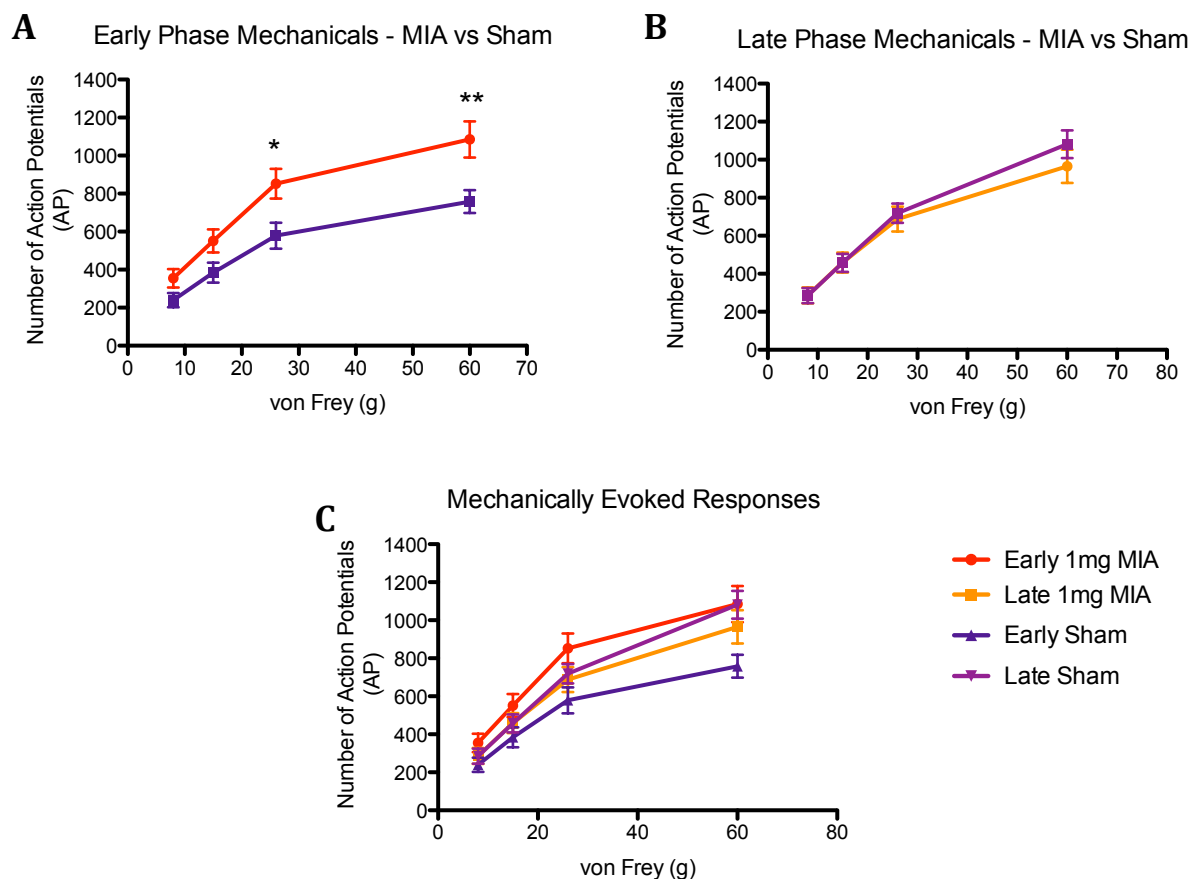


Figure 3.5 - Comparison of mechanically evoked responses of Lamina V WDR cells in MIA and Sham animals at either Early or Late stages after injection. A) Early MIA animals exhibited significantly enhanced evoked responses to punctate mechanical stimuli versus Early Sham animals (** $p\leq 0.01$; * $p\leq 0.05$) (Sham $n=13$, MIA $n=14$). **B)** No difference was observed between Late MIA and Late Sham animals in response to punctate mechanical evoked responses. **C)** Comparison of mechanically evoked responses across all animal groups – no significant difference was observed between Early vs. Late MIA, Late MIA vs. Late Sham or Early vs. Late Sham.

however a trend to enhanced evoked responses in Late Sham animals versus Early Sham was seen though this wasn't significant (2way ANOVA; $p=0.088$). When comparing all baseline mechanically evoked responses across all four groups of animals, it seems apparent that there is a trend to enhanced evoked responses in MIA animals but that this is not distinguishable between the late groups as Late Shams exhibit a similar enhancement in evoked responses, where the Early stage MIA animals exhibit the greatest enhancement of WDR evoked responses. As such, while the MIA model appears to provoke a greater than normal response relationship to punctate stimuli at both time points, the sham model additionally seems to develop an mild increased response to punctate stimuli in the later days after injection of saline that does not exceed that produced by MIA.

Thermally Evoked Responses

The evoked responses of Lamina V WDR cells following the application of thermal water jets to the receptive field in the IL paw produced a stimulus-response curve, whereby the increasing temperature evoked a greater number of action potentials. Two-way ANOVA found that this stimulus response relationship was significantly enhanced in Early MIA animals compared to their Sham counterparts (Figure 3.6A: $p=0.018$), where Bonferroni post hoc tests indicated that these evoked action potentials were significantly greater in response to water jets at the temperature 35°C and 48°C (Figure 3.6A: * $p\leq 0.05$), innocuous and strongly noxious respectively. This trend was strongly observed across all temperatures in the Early MIA animals.

No difference was observed in the responses of Late MIA and Late Sham animals (Figure 3.6B), nor between Early MIA and Late MIA (2way ANOVA; Figure 3.6c). Notably however, a significantly greater number of action potentials were evoked by thermal stimuli in the Late Sham compared to Early Sham (2way ANOVA $p=0.018$), where Bonferroni post hoc tests indicated that these evoked action potentials were significantly greater in response to water jets at the temperature 35°C and this trend carried across the temperatures (Figure 3.6C; # $p\leq 0.05$). As observed for the mechanically evoked responses, there is an observable trend by which all MIA animals and Late Sham responses are greater than those observed in the Early Sham in response to thermal stimulation. As such, while the MIA model appears to provoke a greater than normal response relationship to thermal stimuli at both time points, the sham model additionally developed an increased response to thermal stimuli in the later days after injection of saline.

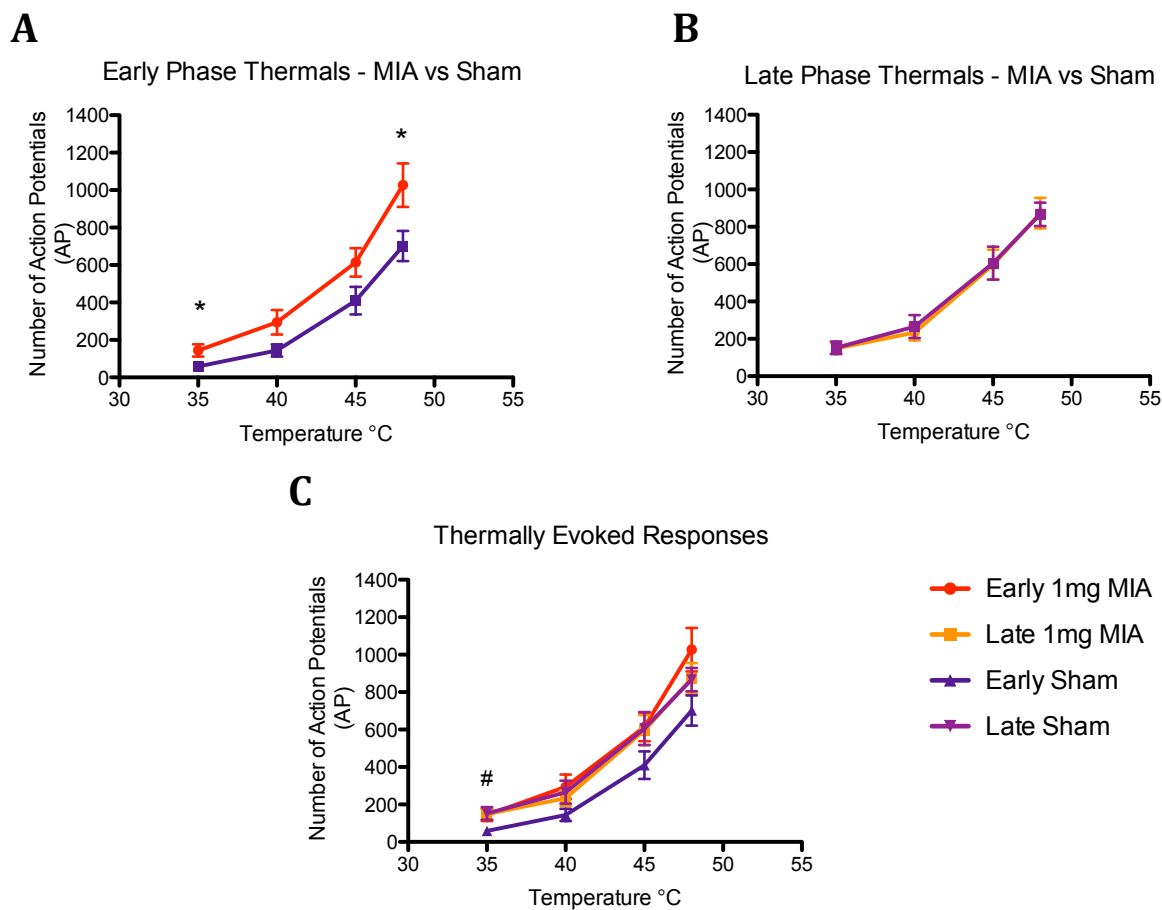


Figure 3.6 - Comparison of thermally evoked responses of Lamina V WDR cells in MIA and Sham animals at either Early or Late stages after injection. A) Early MIA animals exhibited significantly enhanced evoked responses to thermal stimuli versus Early Sham animals (* $p \leq 0.05$) (Sham $n=13$, MIA $n=14$). **B)** No difference was observed between Late MIA and Late Sham animals in response to thermal water jet (Sham $n=15$; MIA $n=14$). **C)** Comparison of mechanically evoked responses across all animal groups – no significant difference was observed between Early vs. Late MIA, Late MIA vs. Late Sham or Early vs. Late Sham. ($n=13-15$)

3.4 Discussion

The MIA model of OA is one of the most common rat models of OA (Little and Zaki 2012), as a result of the ease of induction, reliable histopathology and pain like hypersensitivities observed across both behaviour and electrophysiology. However, the doses most commonly utilized are $\geq 2\text{mg}$ (See Thakur et al 2012, Table S2 for a summary), doses that have recently been identified as provoking neuropathy and a pain state that may only represent a specific subset of patients. The aim of this chapter was to confirm a behavioural and electrophysiological profile indicative of the induction of an OA like pain state in these animals at a dose of MIA that does not cause neuropathy.

Here we demonstrate that in the 1mg MIA model animals develop both a punctate mechanical hypersensitivity at the paw and a shift in weight distribution from the ipsilateral limb, at both the early and late stages of the model versus sham animals. Similarly, electrophysiology in these animals identified increased activity in response to both dynamic brush, punctate and thermal stimulation of the hind paw in the Lamina V WDR cells of Early MIA animals compared to Early Shams. While similar differences were not identified between Late MIA and Late Sham, it seems possible that this may be due to the increased baseline responses observed in Late Shams compared to Early Shams, to both punctate and thermal stimuli.

3.4.1 Behavioural Hypersensitivity

Shifts in weight bearing are indicative of established joint discomfort

The assessment of weight bearing as a measure of joint discomfort in the study of OA is common. It is well established at both 2mg and 1mg MIA doses that a shift occurs onto the contralateral paw which peaks in the early days of the model, and becomes consistent around 10 days after injection (Bove 2003, Pomonis, Boulet et al. 2005, Kelly, Dunham et al. 2012, Thakur, Rahman et al. 2012), where no difference was observed between these two doses (Thakur, Rahman et al. 2012). The present work confirms this, demonstrating that at both time points the 1mg MIA model produced a shift in percentage weight borne not observed in shams at the equivalent time point, where there was no difference observed between day 3 and day 10 in MIA animals.

Theories differ as to the exact relation of weight bearing deficits to clinical pain in OA. Many equate this measure to a primary hypersensitivity or mechanical allodynia of the joint, where the normally innocuous use of the knee, namely placing pressure on it, becomes painful, resulting in weight being shifted to the unaffected side. This discomfort could result from either peripheral or central

sensitization mechanisms, though the rectification of this shift in weight bearing by NSAID therapy suggests a significant contribution of peripheral, inflammatory factors(Bove 2003, Pomonis, Boulet et al. 2005). Others, such as Kelly et al 2012, suggest that these weight bearing deficits correlate to increased spontaneous C fiber activity in these animals, both indicative of a peripheral sensitization but also an increased likelihood of central sensitization(Kelly, Dunham et al. 2012). As such, this suggests the measure equates more to pain at rest or ongoing pain(Mogil and Crager 2004), also observed in patient pools(Hunter, McDougall et al. 2008).

Secondary Mechanical Hypersensitivity, But No Cooling Hypersensitivity

Though changes in weight bearing are informative measures of joint discomfort, they do not provide discriminatory evidence as to the mechanism underlying the pain observed – which is likely a combination of both peripheral and central sensitization. Reports of mechanical hypersensitivity in the paw however provide confirmation of a secondary hyperalgesia, or referred pain, which are suggested to characterize the presence of central sensitization, something commonly observed in the clinic (See Section 1.4.4) . While well described in the 2mg model of MIA, fewer studies have described this in the 1mg model(Sagar, Staniaszek et al. 2010, Thakur, Rahman et al. 2012). Here we demonstrate that at both days 3 and 10 following the injection of 1mg MIA animals exhibit increased sensitivity to vF8g punctate stimulation to the ipsilateral hind paw compared to time matched sham controls. However, no differences were observed at vF1g, vF6g or to Acetone.

While these behavioural changes are broadly in line with expectations, based on previous studies demonstrating secondary mechanical hypersensitivity in MIA animals (Bove 2003, Fernihough, Gentry et al. 2004), this observed hypersensitivity did not extend to vF6g or vF1g as observed in both 1mg and 2mg MIA models previously(Rahman, Bauer et al. 2009, Vonsy, Ghandehari et al. 2009, Burnham 2012, Thakur, Rahman et al. 2012). The overall mean difference in number of withdrawals are also slightly low compared to past literature for 1mg MIA(Thakur, Rahman et al. 2012), where it is hard to draw comparisons with data which use PWTs (e.g. Fernihough et al 2004). Similarly, these animals did not exhibit cooling hypersensitivity, denoted by withdrawals from acetone application, which are reported in past literature(Rahman, Bauer et al. 2009, Burnham 2012, Thakur, Rahman et al. 2012). Once again, as with punctate hypersensitivity, Thakur et al demonstrated a strong cooling hypersensitivity in a 1mg model, so these differences cannot simply be dismissed as an effect of dose.

These contradictions in behavioural findings are difficult to explain, especially when differences arise within the 1mg model. In part, the dose and time point investigated can explain differences, since the smaller dose vs. 2mg studies and day 10 assessments may mean that joint pathology at day 10 and

extent of inflammation at day 3 are less severe, producing a milder pain profile in these animals(Guingamp, Gegout-Pottie et al. 1997, Sagar, Staniaszek et al. 2010, Thakur, Rahman et al. 2012). However, this does not explain differences between the results here and Thakur et al 2012, which used the same dose and similar time points. Consider specifically cooling hypersensitivity. While many studies demonstrate increased withdrawals from acetone this is not universal – since Vonsy et al 2009 failed to define a cooling hypersensitivity at day 3, day 11 or day 14(Vonsy, Ghandehari et al. 2009). It is possible that these discrepancies are explained by individual experimenter differences, ranging from exact criteria for a response; method and location of acetone or vF application; acclimatization time; MIA injection technique and the subsequent extent of leakage from the joint or other factors. All of these may differentially impact upon behavioural outcomes. Additionally, it is possible that repeated pain testing over numerous days could act as a conditioning stressor that enhanced pain like behaviour, given Thakur et al tested multiple days - where as each rat only experienced one round of behaviour in this protocol. As such, experimental design and execution could explain these differences.

At a more basic level, the gender of the experimenter has previously been shown to adversely affect the accuracy of behavioural protocols and exhibition of pain behaviour in mice, where the scent of a male caused stress related reductions in pain behaviour(Sorge, Martin et al. 2014). Though, in direct contradiction, stress has also been linked to hyperalgesia in rodents(Imbe, Iwai-Liao et al. 2005), so it is possible male experimenters could themselves induced increased behavioural responses if their presence was an environmental stressor. As such, differences in behaviour may occur due to individual experimenter factors as simple as gender and olfactory signals.

It is also worth considering the limitations of the use of mean differences to measure of the number of withdrawals to assess changes in mechanical sensitivity in the paw. As discussed previously, it is not unknown for OA to induces changes in contralateral somatosensation(Creamer, Hunt et al. 1996), likely driven by central sensitization derived changes in descending controls. As such, this measure may overlook contralateral changes that in themselves are informative to elucidating mechanistic contribution to OA pain, but may similarly mask the extent of change in the ipsilateral paw. It is for this reason that in later projects, a paw withdrawal threshold was instead calculated using methodology described by Chaplan et al 1994

Though the above highlight disparities in the extent of the behavioural profile of 1mg MIA model of OA, there is a consistent suggestion of secondary mechanical hypersensitivity in the ipsilateral paw, consistent with the characterization of both this model and the clinical OA profile.

The importance of time point?

Past work has characterized the pain behaviour in the MIA model as biphasic based on studies of weight bearing(Bove 2003, Fernihough, Gentry et al. 2004, Pomonis, Boulet et al. 2005, Kelly, Dunham et al. 2012), nocturnal activity(Guingamp, Gegout-Pottie et al. 1997) or secondary mechanical hypersensitivity of the paw(Vonsy, Ghandehari et al. 2009, Ferland, Laverty et al. 2011, Burnham and Dickenson 2013). As such, this describes behavioural hypersensitivities at both the joint and secondary sites as having a bi phasic profile, broadly as showing an early climax in the first 2-3days following iodoacetate injection, followed by a brief period of respite to day 7, and then a gradually climbing profile by Day 10-14 onwards.

This profile is attributed to the time course of joint pathology exhibited in the MIA model of OA. As discussed above, the initials days following injection are coupled to reductions in proteoglycan(Guingamp, Gegout-Pottie et al. 1997, Janusz, Hookfin et al. 2001), chondrocyte death and cartilage thinning (Dunham, Hoedt-Schmidt et al. 1992, Janusz, Hookfin et al. 2001, Guzman, Evans et al. 2003), plus significant inflammation – involving thickening of the synovium and fat pad, swelling, monocyte and neutrophil infiltration(Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Clements, Ball et al. 2009), TNF α IL-6 and NGF are up-regulation in the joint and SP is increased in the synovium(Ahmed, Li et al. 2012). In line with the timelines observed in behaviour, this inflammation is largely resolved by day 7, peaking during days 1-3(Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Clements, Ball et al. 2009). As such, this first wave of hypersensitivity is attributed to peripheral mechanisms, where the inflammatory milieu not only directly activate afferents serving the joint but also serve to sensitize them, such that there is an observable increase in their responses, decrease in their stimulus threshold and fiber recruitment of previously silent nociceptors (Schaible and Schmidt 1985, Grigg, Schaible et al. 1986, Schaible and Schmidt 1988). This peripheral sensitization is confirmed both by the fact MIA animals exhibit marked hypersensitivity to ACh, BK and 5HT(Okamoto and Atsuta 2010), but also by the efficacy of NSAIDs to quench the pain associated with these initial days of MIA induction(Fernihough, Gentry et al. 2004, Vonsy 2008). This peripheral sensitization similarly drives central changes in sensitivity, facilitating synaptic transmission from neighbouring, uninjured tissue too.

Beyond day 7, studies report the increasing prevalence of joint pathology namely the involvement of subchondral bone(Guzman, Evans et al. 2003), cartilage fibrillation and ossification, osteophytes, bone sclerosis and bone exposure by days 14-21(Dunham, Hoedt-Schmidt et al. 1992, Janusz, Hookfin et al. 2001, Morenko, Bove et al. 2004). As such, beyond day 7 there is a building pathology that begins to activate the rich density of mechano-receptive nerve endings in the bone, to cause an increased information load from the peripheral fibers to the spinal cord upon movement. This is clearly

demonstrated by work directly recording from peripheral afferents 14 days after MIA induction, at doses as small as 0.3mg, where joint movements evoke significantly enhanced firing profiles (Schuelert and McDougall 2009). There is similarly increasing spontaneous activity in mechanosensitive C fibers at these later stages following MIA (Kelly, Dunham et al. 2012), which itself may also help drive central sensitization. This bombardment takes up the space left by the previous inflammation to maintain or reinitiate central sensitization in these animals – though it is likely both this enhanced nociception and the effects of inflammation overlap.

As such, we expect pain behaviour at both these time points, but with pain driven by different underlying mechanisms – the significance of which may become more apparent following pharmacological manipulation of descending controls in Chapter 4. In both instances, there is an expectation that the peripheral barrage would generate a central sensitization sufficient to evoke hypersensitivity in the paw, as well as the ongoing discomfort of the joint, as previous research had lead us to expect (Vonsy, Ghandehari et al. 2009, Burnham 2012, Thakur, Rahman et al. 2012). This was confirmed in this study, where no difference was observed in either the weight bearing or punctate mechanical hypersensitivity profiles of 1mg MIA animals between days 3 and 10 – where both time points were significantly different from saline sham animal responses.

Secondary Hypersensitivity as an Indicator of Central Sensitization

The presence of increased cutaneous sensitivity to vF8g in the IL paw of MIA animals at both time points is taken to be indicative of central sensitization in this model of OA. Put simply, in the absence of damage or inflammation at the paw there are no peripheral changes in the afferent activity that can explain increased sensitivity to this punctate stimulation. Instead, this behaviour is explained by changes in the sensitivity of the neurons of the dorsal horn, to which primary afferents synapse, notably a reduced threshold to activation and increased outputs once activated. Traditionally a barrage of nociceptive information from the periphery triggers this state, but given the paw is undamaged the population of dorsal horn neurons which are primarily served are not subject to such a barrage. There are two possible explanations for this – either the expansion of receptive fields that characterizes a secondary hyperalgesia, or the referral of pain.

In the case of receptive field expansion, it is postulated that ascending fibers serving the joint largely receive inputs from the deep tissues but also receive weak inputs from adjacent cutaneous sites and muscles – inputs that do not usually generate responses from the ascending fiber. Following sensitization, the threshold of activation of this fiber is lowered such that the weak inputs from these neighbouring regions become suprathreshold, thus expanding the receptive field of the cell such that

paw inputs are also transmitted and interpreted as noxious. This is much like the mechanism by which capsaicin generates mechanical hyperalgesia in adjacent sites(O'Neill, Brock et al. 2012).

Alternately, this behaviour could be explained by the referral of pain. Competing theories suggest this may either result from convergence, where co-localization of fibers as they enter the spinal cords allows cross talk that facilitates peripheral inputs from neighbouring tissue; or a central sensitization spreads to adjacent dorsal horn segments, through a combination of interneuronal signaling, the release of inflammatory and algogenic mediators in the spinal cord, in addition to alterations in descending controls.

Joint, muscle and cutaneous afferents may converge in the dorsal horn(Schaible, Schmidt et al. 1987). Similarly, the use of intra-articular tracers confirm projections to levels L3, L4 and L5 DRG/sympathetic ganglia(Edoff, Grenegard et al. 2000), where paw afferents project to L5. As such, cutaneous paw inputs could feasibly synapse onto common neurons with the joint, as suggested in capsaicin studies at the knee(Schaible and Richter 2004). On the one hand, these support the contribution of the expansion of receptive fields in the observed hypersensitivity at the paw during the MIA model. However, the expansion of receptive fields following capsaicin can be further enhanced by spinalization(Neugebauer and Schaible 1990, Vanegas and Schaible 2004), highlighting the important contribution of descending controls, and thus brain stem sensitization, in this process. It has similarly been shown that in models of kaolin and carrageenan induced acute OA that the release of PGE₂ is increased and COX-2 up-regulated in the spinal cord(Ebersberger, Grubb et al. 1999), which would serve to assist the spread of central sensitization. Similarly, the enhancement of Lamina V WDR cell responses during the MIA model contribute to a picture of spreading central sensitization across dorsal segments in this model.

Regardless of the exact mechanism, the contribution of a central sensitization to the observed paw hypersensitivity is clear. That the hypersensitivity observed in the above experiments is perhaps weaker than that observed previously may be accounted for by the activity dependence of central plasticity. This is the concept that the magnitude of the peripheral drive goes some way to determining the extent of change in the balance of central processes, best observed in the adaptations in descending controls discussed in Chapter 4.

Central Sensitization is a key feature of the clinical profile of OA, in which patients exhibit referred pains, increased responses to hypertonic saline injections to adjacent muscle and altered contralateral VAS scores in response to local anaesthesia at the ipsilateral site(Creamer, Hunt et al. 1996, Bajaj, Graven-Nielsen et al. 2001, Kean, Kean et al. 2004). This behaviour, by confirming central sensitization in the 1mg model, confirms the use of this dose to model OA pain.

3.4.2 Sensitization Observed in Spinal Electrophysiology

In assessing the evoked responses of lamina V WDR cells serving the ipsilateral hind paw, these electrophysiology measurements aim to ascertain the presence of a central sensitization in the 1mg MIA model. As discussed above in regards to the mechanical hypersensitivity to vF8g, changes in behaviour and baseline electrophysiology from the paw must rely upon central changes given the absence of a peripheral driver. The key advantage of this measure over behaviour, which it could be argued had already demonstrated the presence of central sensitization, is the ability to assess changes in the responses to suprathreshold stimuli. The data presented here has demonstrated a clear increase in baseline responses of WDR neurons to both thermal and mechanical stimuli in the early stages of the MIA model compared to their time matched sham counterparts. That this difference is not observed in the late stage MIA animals may be the result of either the increased baselines observed in late stage sham animals to which these Late MIA animals were compared, or the lack of central sensitization, or a combination of both. These possibilities will be discussed further below.

Enhanced Responses to Mechanical and Thermal Stimulation at Days 3-5 Following MIA

Rats at days 3-5 following the intra-articular injection of 1mg MIA exhibited heightened evoked responses to dynamic brush, punctate and thermal stimuli compared to early shams, an effect which was greatest in response to the most noxious stimuli: vF26g, vF60g and 48°, demonstrating the development of a hyperalgesia in these Early MIA animals. Though the response of these animals to vF8g is not considered significantly greater than shams, the p value of 0.077 and trend observed here towards heightened responses compliments the observations made in behaviour. No significant difference in the responses of these animals to electrical stimulation was observed indicating a lack of changes in excitability of peripheral nerves innervating the hindpaw, which further supports central changes as a basis for the IL paw changes.

These observations are almost exactly aligned to those from previous work in this lab at days 3-5 of the 2mg MIA model, which reported significantly heightened responses to mechanical and thermal stimulation at vF60g and 48°C (Burnham 2012). Notably however, that study also reported significant increases in evoked electrical responses, notably in the number of action potentials attributed to each of A β , A δ and C fibers, increase input, but no difference in dynamic brush at this time point of the 2mg MIA model—unlike the findings presented here. While electrical evoked responses are not considered significantly different in the present study, it can be observed that there is a trend towards enhanced A δ and C counts in both the MIA groups and the late sham animals compared to early sham. This relationship is significant for the comparison between Late MIA and Early Sham (p=0.037, unpaired T-

test) and nears significance for Early Sham vs. Late Sham ($p=0.074$, unpaired T-test). As such it may be the extent of change in electrically evoked responses are less pronounced here, but still present in the early (and late!) stages of the 1mg MIA model.

While this allows clear conclusions to be drawn about the presence of a central sensitization that facilitates both punctate and thermal sensation in the early stages of the MIA model in both 1mg and 2mg doses, conclusions explaining the observed differences in brush responses are more complicated.

Dynamic brush allodynia is considered a strong marker of central sensitization (Latremoliere and Woolf 2009). The presence of heightened evoked responses to dynamic brush in Early MIA but not Late MIA or sham animals in this work would normally suggest the presence of a strong *initial* central sensitization that weakens at later stages in the model with the decline on inflammation. However, enhanced brush responses are not observed in the early days of the 2mg model contradicts this theory (Burnham 2012), given that it is known the behavioural profile is much greater with increasing doses, even in the early days (Guingamp, Gegout-Pottie et al. 1997, Sagar, Staniaszek et al. 2010, Thakur, Rahman et al. 2012). The challenge is that very few studies characterize dynamic allodynia in behaviour or at this early stage of the MIA model with electrophysiology. While not considered significant, in a behavioural study of the 2mg model a temporary decrease in the PWL to cotton bud stroke was observed at day 7 of the model, but not later time points (Combe, Bramwell et al. 2004). Unfortunately, this was the earliest time point investigated but does suggest that behaviorally there could be a very short term dynamic allodynia in MIA animals as a result of the induction of the model.

Inferences can be made from other pain models, where a brush allodynia has been characterized in secondary areas following capsaicin (Andersen, Gracely et al. 1995). Because brush sensations are reliant upon A β inputs which do not express the capsaicin receptor TRPV1 and nor can this brush allodynia be relieved by topical NSAIDs, this dynamic allodynia are attributed to the presence of central changes in the spinal cord that allow A β inputs to be transmitted as nociceptive signals (for a full review and discussion of this effect see (O'Neill, Brock et al. 2012)). These observations are similarly made for evoked brush in WDR recordings in a UVB rekindling model (Jessica O'Neill* 2015). In each of these instances, changes to responses to dynamic brush are driven by a strong peripheral drive. It is possible that the initial inflammatory phase of this 1mg MIA models could provide a similar drive, and thus the observed changes. This matches well to observed enhancements to punctate and thermal stimuli too. However, it is important to consider the inconsistency in evoked brush responses between pharmacology cohorts, discussion in Section 3.4.3. While this might be taken to suggest inconsistencies in success of induction of the MIA model in some animals, the differences may best be explained by considering differences in sham responses (Figure 3.7) masking a potential significant brush allodynia in the ondansetron cohort MIA rats.

The suggestion that these heightened responses from the paw in electrophysiology may be driven by inflammation at the knee at days 3-5 is hard to validate, given the relative lack of electrophysiology focusing on this time point in the model. That both this study and Burnham et al 2012 exhibit these mechanical and thermal enhancements in these early days of MIA model confirms the presence of central sensitization at this stage of the model(Burnham 2012), where validation of the contribution of inflammation to this must instead be provided by behavioural studies focusing on paw sensitivities. One clear example is given by Vonsy et al 2008, where the administration of methylprednisolone on day 0 of the induction of the MIA model significantly attenuated the hypersensitivity expressed on days 3 through to 7(Vonsy 2008). Beyond this point, the paw hypersensitivity regained to a point similar to untreated MIA animals. This clearly demonstrates the importance of the inflammatory response, thus supporting the proposal that the observed changes at days 3-5 in the MIA animals relates to an inflammation driven central sensitization.

Evoked responses are unchanged in the later stages of the MIA model of OA

At days 10-14 following a 1mg injection of MIA, the responses of Lamina V WDR cells are no different to their time matched counterparts, who received just saline, in response to both electrical, brush, punctate mechanical stimulation and water jet. In fact, the results are practically identical (Figure 3.5b and 3.6b). While the idea that MIA may not provoke enhancements of evoked responses at day 14 onwards is nothing new, there is no clear-cut consensus – even within the 2mg MIA studies in this lab. While two characterize a significant increase in evoked responses to punctate stimuli in rats at day14+ following 2mg MIA, in agreement with work in mice, others have failed to replicate any significant alteration in baseline responses at day 14 of this 2mg model(Vonsy 2008, Patel 2012, Thakur 2012). Rahman additionally characterized an increased mechanically evoked after discharge in WDRs at days14 onwards in the 2mg MIA model(Rahman, Bauer et al. 2009). As such, there are clearly two distinct “camps” – one in which there is no clear central sensitization, and one where there is.

Beyond this lab, Sagar et al 2010 describes no alteration in the responses of WDRs in either 1mg or 3mg MIA treated rats at days 14-17 to punctate stimulation, but outlines a significant sensitivity to vF10g and vF15g by days 28-31(Sagar, Staniaszek et al. 2010) – though a relatively small number of neurones were characterised. Crucially, at days 28-31 the enhancements in evoked responses to punctate stimulation are strongly correlated to changes in cartilage, synovium and subchondral bone. This brings forward the first possible explanation for these differences – the extent of model progression, disease pathology and thus nociceptive drive from the knee joint may differ. Perhaps in

those studies exhibiting central enhancements, namely enhanced evoked responses of WDRs, the pathology has progressed further. As previously described, higher doses of MIA to the knee provoke faster and more severe joint pathology (Guingamp, Gegout-Pottie et al. 1997, Pomonis, Boulet et al. 2005, Thakur, Rahman et al. 2012), where there is a concentration dependent enhancement of peripheral afferent responses to noxious movement of the joint (Schuelert and McDougall 2009) and ongoing afferent drive (Okun, Liu et al. 2012). In my own study of the 1mg MIA model at days 10-14 we would expect joint pathology to be much less severe and the extent of peripheral drive from the joint to be reduced, while individual differences in injection techniques across different studies could perhaps affect the extent of pathological progression in the 2mg experiments (e.g. leakage). As such, it could be argued that the drive from the joint is insufficient to provoke central sensitization in these animals, hence the lack of difference in baselines from shams.

However, this then leaves the question as to why we observe an increased sensitivity to vF8g in behaviour at this time point. The picture is complicated – it may be that changes in paw withdrawal behaviour are more reliant upon changes in Lamina I WDR cells, where Thakur described enhanced A δ input during electrical stimulation and heightened evoked responses to vF8g in 2mg MIA Lamina I WDRs, despite describing no changes at Lamina V. These Lamina I WDRs additionally showed enhanced receptive fields (Thakur, Rahman et al. 2012). Here he argued that changes in Lamina I drive behavioural hypersensitivity, where changes at Lamina V excitability were not observed as a result of the more complex informational integration and dilution – including interneuron modulation by glycine and GABA; descending modulation, triggered by ascending signals from lamina I; and the fact Lamina V WDRs receives inputs from A β 's that Lamina I does not. Also, it is worth noting that behaviour observed in my study is notably milder than observed in previous 2mg work – as such it may be that Lamina I is driving this milder sensitivity but that as model severity increases, Lamina V similarly becomes sensitized to provoke more significant behaviour.

Alternately, as discussed above, behavioural changes may be the result of the expansion of receptive field, a secondary hypersensitivity, as opposed to spreading sensitization to those segments serving the paw investigated during spinal electrophysiology. It may be that while Lamina V WDR neurons which predominately serve the hind paw do not exhibit enhancements during this stage of the 1mg model - perhaps as a result of differential changes to descending control – those ascending fibers serving the knee do become sensitized, and thus respond to the previously sub threshold inputs at the edges of their receptive field. That these broad but often silent fields, extending from the knee to the paw, exist has been demonstrated by Sagar et al 2015. This study examined the activity and receptive fields of WDR neurons serving the joint in arthritic animals, 21 days after they received 1mg MIA. They demonstrated that following the intra articular injection of NGF, the Lamina V cells of MIA rats, but not shams, exhibited both enhanced firing and >100% increase in the receptive field of this cell, extending

down to include the paw(Sagar, Nwosu et al. 2015). Critically, this study suggests that peripheral changes in the afferents serving the knee, including up-regulation of TrkA, drive spinal sensitizations that facilitate an expansion of the area over which sensory inputs are engaged. In other words, while late MIA animals may not exhibit evidence of central sensitization at the segment serving the paw, sensitizations are likely present at higher segments serving the knee that can account for punctate hypersensitivity at the paw.

However, considering the data presented in Figure 3.5c and 3.6c, another distinct explanation for the lack of observed excitability in late MIA animals presents. It may be that the evoked responses are not considered heightened because of changes in the baseline responses of Late Sham animals. In other words, an enhancement of the baselines on Late Shams (when compared to Early Shams) may be masking an effect when compared to Late MIA. Consider the evoked responses to punctate mechanical stimulation: there is a strong trend suggesting Late Sham baselines are greater than those in Early Sham rats ($p=0.088$). For thermal responses, this difference in evoked response *is* significant, at $p=0.018$. The overlay of baselines in these two graphs, Figure 3.5c and 3.6c, clearly demonstrates enhancements of both MIA groups and Late Sham responses above those seen in Early Shams. In this same vein, where we have considered the Early MIA baselines to be more excitable compared to shams, thus demonstrating central sensitization, these responses are not significantly different from late MIA animals, for either punctate or thermally evoked responses.

As such, it may be that as opposed to an absence of central plasticity in the Late MIA model, there have additionally been changes observed in the sham model as a result of the injection procedure itself. It could be hypothesized that the insertion of a needle to the intra articular space could cause physical damages, not least if the needle passes through the patellar tendon, to weaken it and cause long term destabilization of the joint, or scratches the surface of the articular cartilage to leave a lesion that could cause friction. Similar effects have been observed in sham animals in work from other labs, where paw withdrawal thresholds declined in shams following injection – though the declines were much greater in MIA animals in this study, as expected(Mapp, Sagar et al. 2013). It may be in that at day 10 of a 1mg model, joint pathology changes are not significant enough drivers of pain to differentiate from changes occurring as a result of the injection procedure itself.

The best way to gain clarity on these changes in the 1mg MIA model would be further study. Collecting electrophysiology baselines for naïve animals, and 1mg 21day+ animals, could elucidate further the extent of excitability changes in the dorsal horn and the role of extent of joint pathology, or lack thereof, on evoked responses in this MIA model of OA.

3.4.3 Study Limitations

In considering the results and potential conclusions of this chapter, it is important to consider the limitations of the present study's design – both to provide context that will inform the conclusions, but also suggest potential modifications to the study that would have allowed greater clarity.

Evidence of Structural Pathology

A major limitation in the discussion of the results presented in this chapter, as in all the chapters of this thesis, is the absence of analysis of the structural pathology of these animals. While previous literature has established the histopathological changes associated with both the early (Strassle, Mark et al. 2010, Kelly, Dunham et al. 2012) and late (Ivanavicius, Ball et al. 2007, Kelly, Dunham et al. 2012, Thakur 2012) stages of a 1mg MIA model, it would be a fallacy to assume that every injection of MIA was successfully delivered. Even in a clinical setting, IA therapies commonly miss their mark (in much larger knees) without ultrasound guidance (Berkoff, Miller et al. 2012). There are similarly considerations of the potential off-target effects of MIA, such as leaking from the synovial joint to affect surrounding tissues and drive pain independently of OA-like pathology – including potential uptake by local neuronal endings to trigger neuropathic pain. These possibilities undermine the assumption that a pain profile necessarily demonstrates a successful injection and the presence of OA-like changes in the knee joint.

Such variability of success of injections might explain the inconsistency of the presence of a significant enhanced evoked responses to dynamic brush observed in the baseline responses of the ondansetron treated and atipamezole treated cohorts of early 1mg MIA and sham rats (Compare Figure 3.4b, 4.1b and 4.3b).). I have summarized this is Figure 3.7. When baselines of these early MIA animals are merged a significant enhancement is observed to dynamic brush versus sham animal evoked responses. While this significant effect was observed in the atipamezole treated cohort's baselines the effect was not observed for the ondansetron cohort baselines. This suggests a different profile between the two sets of 1mg MIA treated rats, perhaps where significance in the

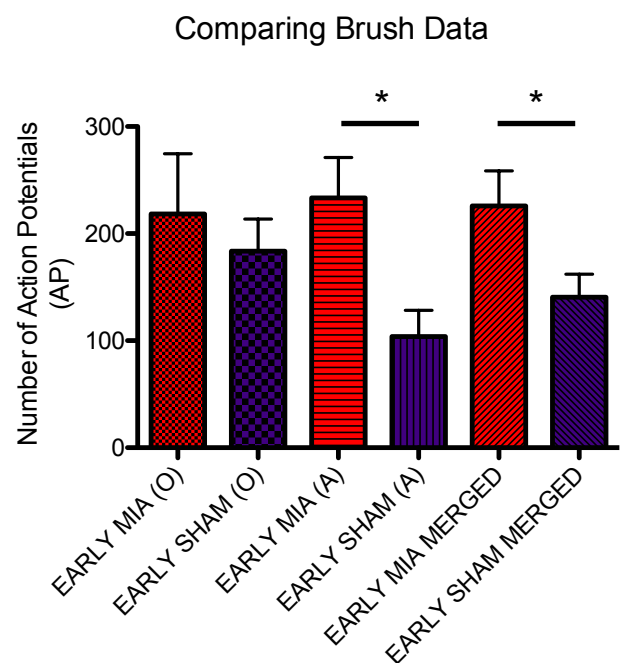


Figure 3.7 - Comparison of dynamic brush evoked responses of Lamina V WDR cells in Early MIA (red) and Early Sham (purple) animals depending on the experimental cohort. Those animals used in the ondansetron experiments (O) show no significant difference in baseline evoked activity to brush, unlike the atipamezole cohort (A), which may in turn drive the significance observed in the merged cohort (* $p \leq 0.05$)

merged analysis is driven by the dramatic difference in the atipamezole treated cohort's baselines. An understanding of the differing structural pathophysiology, or off target effects due to failed injections, including potential physical damage that could cause a post-op style sensitization in some sham animals, could provide clarity of potential mechanisms underlying this difference that the currently presented data alone cannot. However, it appears from Figure 3.7 that in reality these between groups are driven by differences in the extent of the Sham animal response to dynamic brush – with relative consistency in the extent of evoked activity between the MIA Early groups. This might suggest this is not a problem of failed injection at all, but of insufficient n numbers – discussed further in the Chapter 4's study limitations – and a degree of variability of WDR profiles.

The conclusions drawn in this study (Chapters 3 and 4) are based upon potential considerations of the extent of histopathological changes – inflammatory in the early animals, and structural in the late animals. These conclusions would be far stronger with a) evidence of such changes, on which to base these conclusions and b) a correlation analysis, to detect any potential relationship between the extent of structural pathology, behaviour and electrophysiology. Of note, previous analysis of this kind has failed to characterize a relationship between structural pathology and nociceptive behaviour or electrophysiology (McDougall, Andruski et al. 2009, Kelly, Dunham et al. 2012), suggesting alternate explanations for the differences observed between this work and earlier studies in the 2mg MIA could be required.

Such analysis could also allow the exclusion from analysis of animals in which OA had not been successfully induced by the MIA model. It is possible that in so doing, more significant differences between groups and after pharmacology might be detected. Conversely, in a meta analysis of studies that measured behavioural pain outcomes in small animal models of OA, Suokas et al 2014 demonstrate that *“Lack of reported evidence that OA structural change was successfully induced in the model was strongly associated with larger effect sizes”*, where effect size refers to reported analgesic efficacy (Suokas, Sagar et al. 2014). This analysis suggests that incomplete phenotyping of animals, including the failure to confirm structural pathology, prior to pharmacological interventions may lead to false conclusions. In this instance, the false conclusion would be to attribute differences in the response to ondansetron or atipamezole in these 1mg animals vs. previous 2mg studies to structural pathological differences, when these differences may instead related to off target effects of MIA in tissues surrounding the joint capsule

Direct Comparisons Between 1mg and 2mg Animals

In opting not to include a 2mg MIA cohort within these studies, I had sought not to replicate work already published and to instead use it as a comparator reference (Rahman, Bauer et al. 2009, Burnham

and Dickenson 2013). However, this approach may be flawed given the previously discussed variability and lack of consensus on whether the MIA model results in increased evoked responses to mechanical and thermal stimulation, and the absence of structural pathology analysis in these reference studies (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). Though the use of 2mg MIA cohort for direct comparison would have required the repetition of previous studies, it would have facilitated direct comparison between sham, 1mg and 2mg MIA animals without accounting for inter-experimenter variability. In conjunction with joint histopathology, such work could have allowed analysis of any potential relationship between dose, joint pathology and evoked electrophysiology and pharmacology.

3.4.4 Overall Implications

In conclusion, herein I have demonstrated a significant behavioural profile in the 1mg model of MIA that involves both ongoing pain from the joint, as observed by incapacitance, and a secondary hypersensitivity in the paw, demonstrated by increased withdrawal from vF8g, at both the early and late stages. The combination of this behavioural profile with the changes in excitability observed in Lamina V dorsal horn WDRs at early but not late time points in MIA animals may indicate that this pain is driven differentially at the two stages. During the initial days of the model, inflammation at the joint may cause the spreading of pain to the paw, likely through a combination of the expansion of receptive fields, convergence and spreading central sensitization, possibly due to changes in descending controls. During the later stages of the 1mg model, the mildly enhanced sensitivities at the paw appear to be driven by the expansion of receptive fields. It is likely that over time the pathology in this 1mg model would increase, eventually driving changes observed in these late stages in the 2mg model – notably more emphasized behavioural pain profiles and more excitable electrophysiology baselines. However, all things considered, this may allow the 1mg model at day 10 to effectively model the early years of clinically developing OA pain, and day 3 the periodic flares of painful synovitis experienced by patients.

Chapter 4 – Descending Monoaminergic Controls in the MIA Model

The ultimate goal of modeling OA pain in the MIA model is to better elucidate the mechanisms underlying this chronic pain condition and validate new avenues of therapy. Mirroring the established effects clinically, numerous trials have confirmed and compared the efficacy of NSAIDs, COX-2 inhibitors, capsaicin, opiates and mAbs for NGF in the MIA model(Bove 2003, Combe, Bramwell et al. 2004, Pomonis, Boulet et al. 2005, Ivanavicius, Ball et al. 2007, Kalff, El Mouedden et al. 2010, Ishikawa, Koya et al. 2015). With the clinical efficacy of antidepressants in OA pain becoming increasingly established (see Section 1.3.1), attention has turned to the possible role of descending controls in development and maintenance of pain in the MIA model of OA. By better understanding the role this descending system plays, it is possible that pharmacological intervention can be better tailored to exploit or block these changes and thus provide superior analgesia to the current standard of care.

4.1.1 Serotonin

As discussed in **Section 1.4.6**, serotonin is a pivotal transmitter in the modulation of spinal signaling and is released from fibers descending from the brainstem. Serotonin exerts a bidirectional control on transmission in the spinal cord, by virtue of an interaction with different receptor subtypes. Serotonin at 5HT₇ will inhibit transmission and provide analgesia while 5HT₃ enhances transmission to induce/sustain hyperalgesia(Dogrul, Ossipov et al. 2009) – where the balance of controls may be altered during disease states(Suzuki, Rahman et al. 2004, Suzuki, Rygh et al. 2004, Suzuki, Rahman et al. 2005).

Previous work has utilized the 5HT₃ antagonist ondansetron in a 2mg MIA model of OA, highlighting an adaptive change in the excitatory serotonergic drive modulating low threshold evoked neuronal responses in MIA-induced OA pain(Rahman, Bauer et al. 2009). Specifically, it was shown that ondansetron produced marked inhibition of dynamic brush, punctate and thermally evoked responses applied to the paw, where this brush and non-noxious punctate inhibition was not observed or less robust in animals given a sham injection into the joint (saline)(Rahman, Bauer et al. 2009).

Similarly it has been demonstrated that the inhibitory serotonergic drive may also have a role to play in OA pain. The 5HT₇ antagonist, SB-269970, significantly enhanced both the noxious thermal and electrically evoked responses in late phase MIA animals, an effect not observed in naïve or early phase MIA animals. This suggests that MIA and the joint destruction that develops as a consequence of its injections may induce an increased 5HT₇ mediated serotonergic inhibition as joint destruction

becomes chronic and nociceptive drivers ongoing, perhaps as a counterbalance to the development of central sensitization in this model.

Milnacipran, the serotonin and noradrenaline reuptake inhibitor (SNRI) utilized in the treatment of major depressive disorders, successfully attenuates both mechanical, thermal and electrically evoked responses in MIA animals (Burnham and Dickenson 2013). Crucially, this study found the effect of milnacipran was reversed significantly by spinal SB-269970, indicating that the analgesic effect of this SNRI in the OA pain model is in part mediated by an interaction between descending serotonergic controls and spinal 5HT₇ receptors.

Similarly Tramadol, a μ -opioid receptor agonist and SNRI, has been shown to be highly effective in the treatment of punctate allodynia and weight bearing deficits in the MIA model (Combe, Bramwell et al. 2004), where the efficacy of Tramadol is attributed in large part to the activation of the 5HT₇ receptor (Yanarates, Dogrul et al. 2010).

4.1.2 Noradrenaline

As with Serotonin, Noradrenaline also has a crucial role to play in the modulation of pain signaling in the MIA model –the role of NA in pain is discussed more fully in **Section 1.4.6**. NA descending from the dorsolateral pontine nuclei binds at spinal α_2 adrenoceptors to hyperpolarize cells, reducing both the likelihood of action potential generation but also the release of transmitters at pre-synaptic terminals. Work in both acute and chronic inflammation, and neuropathy, has pointed to a time-sensitive role in the extent of the noradrenergic modulation in pain transmission (Green, Lyons et al. 1998, Pertovaara 2006, Hughes, Hickey et al. 2013).

Relatively little work has been performed on the role of NA in models of OA pain, despite the efficacy of SNRIs like duloxetine to treat OA in the clinic (Chappell, Ossanna et al. 2009, Sullivan, Bentley et al. 2009, Hochberg, Wohlreich et al. 2012, Pergolizzi, Raffa et al. 2013). Work from this lab has investigated the effect of atipamezole, an α_2 antagonist, in a 2mg model of MIA, demonstrating that this drug significantly enhanced the response of WDRs in early phase MIA animals to punctate stimuli, but had no effect in either late phase or naïve animals (Burnham and Dickenson 2013). Interestingly, the administration of spinal atipamezole following systemic milnacipran reversed the inhibitory effect of the latter on responses of deep WDR neurones to both mechanical and thermal stimuli back to pre-milnacipran baselines in the early phase of the MIA model (3 days). However, a full reversal of this inhibitory effect was not seen with spinal administration of atipamezole in the late-phase of the model (Burnham and Dickenson 2013), suggesting a declining role for noradrenergic descending modulation

in the latter stages of this model and thus a role for agents acting upon this descending system that may depend on the state of the OA and/or OA pain.

The combined conclusion from this and the results with SB-269970 above would indicate that the mechanism underlying the analgesia produced by the SNRI milnacipran changes over time during the MIA model – whereby NA at the spinal α_2 adrenoceptor drives the inhibitory effect in the early, inflammatory stages of MIA, while 5HT at the spinal 5HT₇ receptor mediates this inhibition in the latter stages of the model, where pain is driven by advanced pathology and not inflammation.

These spinal electrophysiology studies indicate time-dependent alterations in monoaminergic regulation of the 2mg MIA model. During the early inflammatory stages of the model, there is a descending noradrenergic drive limiting transmission to punctate stimuli in the dorsal horn via α_2 . During the latter stages, there appears to be a 5HT-driven inhibition of thermal evoked responses via spinal 5HT₇ receptors, while an excitatory serotonergic drive enhances low-threshold and thermally-evoked neuronal responses via spinal 5HT₃ receptors. This demonstrates the importance of the balance between these antagonistic controls. It is not yet known if or how the role of spinal 5HT₃ receptor activation changes during the early phase of MIA.

One interesting comparison that can also be made throughout these studies is the relative inhibitory contribution of 5HT or NA to differing peripheral stimuli, perhaps clearest in the work of Burnham et al. The relative extent of reversal of the effect of milnacipran by spinal SB-269970 was much greater for thermally-evoked than mechanically-evoked responses, while spinal atipamezole only enhanced responses of mechanically-evoked activity and not thermally-evoked activity. In other words, descending inhibition from the serotonergic system has a greater effect on thermally-evoked responses, while descending noradrenergic inhibition is more effective in modulating mechanically-evoked responses. This is not a new concept - it has been shown that intrathecal NA is 180 times more potent than 5HT at inhibiting mechanical nociception(Kuraishi, Harada et al. 1979). It has also been shown that the analgesic effect of morphine on different forms of nociception can be similarly divided – descending NA playing a greater role in inhibiting mechanical nociception (e.g. to tail pinch), while descending 5HT played a greater role in inhibition of thermal nociception (e.g. to hot plate), though it was noted that both transmitters had a role in morphine-induced analgesia to both forms of stimulation(Kuraishi, Harada et al. 1983). The consequence of this for OA is that we would expect noradrenergic systems to provide more potent relief of this predominantly mechanical hyperalgesia and allodynia– such that an SNRI would be preferable to an SSRI, although this remains to be empirically tested.

It is worth noting that these aforementioned preclinical studies all focus on stimulation of the paw. Work by Kelly et al 2013 assessed the effect of spinalization on the thresholds and reflex responses of the tibialis anterior (TA) or biceps femoris (BF) in animals given an 1mg intra-articular dose of MIA to the knee joint and those given a similar sham injection at days 14 and 28. While the injection of MIA resulted in reduced mechanical thresholds in the BF, which were unaltered by spinalization at day 28, the effect of MIA injection on mechanical sensitivity in the TA was only unmasked by spinalization – which revealed a hyperexcitability (Kelly, Dobson et al. 2013). This suggests that secondary hyperalgesia at the BF is maintained at the spinal level, not by descending facilitations, while at the TA spinal hypersensitivities are moderated by descending inhibitions. Interestingly, these reflexes were unaltered at day 14, suggesting that central sensitization in this 1mg model manifests at later time points. The implications of this for the investigation of changes in descending control at the paw in a 1mg model is the expectation of enhanced descending inhibitions at a later stage of the model (as seen in the TA), driven by enhanced pathology at the knee, that may not be visible at day 14 but could be evident by day 28. Given the findings by Burnham in the 2mg model discussed above, it could be hypothesized that these descending controls that mask hypersensitivities are serotonergic inhibitions.

Previous work from this lab has identified time-dependent changes in the noradrenergic and serotonergic descending controls mediating spinal excitability, and thus pain perception, in a 2mg model of OA pain compared to sham and naïve controls. Given this dose of MIA has been identified as driving neuropathy (Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012), and the extent of change of descending controls has been shown to be variable between different pain conditions (Green, Scarth et al. 2000, Rahman, Suzuki et al. 2004), it is hypothesized that, at this lower, non-neuropathic dose of MIA, modifications in the descending control on spinal excitability by NA and 5HT, as revealed by the spinal application of various antagonists, may also differ.

4.1.3 Chapter Aims

For patients with symptomatic OA the unmet needs are still clear – efficacious, safe and tolerable analgesia. However, many drugs still struggle to translate from laboratory success to marketing approval for OA pain.

While this problem in part stems from the difficulties in translating pharmacological observations in animal models due to poor study quality (Suokas, Sagar et al. 2014), it could also stem from an over generalization that results in one model of OA pain relate to a broad and diverse patient cohort. Previous work has suggested that the MIA model of OA pain may model a more neuropathic driven OA

pain condition at doses of ≥ 2 mg at 14days, but not the 1mg model(Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012). Thus pharmacological observations identifying dynamic contributions of descending controls in the 2mg model (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013) may relate to a patient segment with neuropathic like pain, and not the larger cohort with non-neuropathic pain.

This study aims to characterize differences in the extent of descending control, both serotonergic and noradrenergic, in the early and later stages of a 1mg MIA model of OA pain versus those previously characterized and published in the 2mg model (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013) – now understood to be neuropathic in nature(Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012). This will be studied using spinal cord electrophysiology and the selective antagonists ondansetron and atipamezole.

4.2 Methods

4.2.1 Animals

All work was conducted in Male Sprague Dawley rats, bred and housed in the Central Biological Services Unit at University College London. As described in Chapter 2, behavioural and electrophysiological studies were conducted either at the early stages of OA pain development, on days 3-5 after injection of either MIA (1mg, 25µl,) or Saline (25 µl); or late stages, on days 10-14 after injection in animals weighing in the range of 220-250g. At the time of experimentation As such, results for four populations of experimental animals are described in this chapter: Early MIA, Early Sham, Late MIA and Late Sham.

4.2.2 Induction of the model

As detailed in Section 2.2, rats were injected with either 25µl l of 1mg MIA (Sigma, UK) or Saline to the left knee.

4.2.3 Behavioural Assessment

Methods and results presented in Chapter 3 (Section 3.2.3 and 3.3.1)

4.2.4 Electrophysiological Assessment

Spinal electrophysiology was carried out in rats weighing 220-250g at either days 3-5 (Early) or days 10-14 (late) after injection of either 1mg MIA or Saline, as described in detail in Section 2.4.

Once a single WDR neuron had been isolated, rounds of 20minute recordings of evoked neuronal responses to electrical, mechanical and thermal stimuli were performed. Once each neuron had produced stable responses to three consecutive rounds of stimulation by the same stimuli set, pharmacology was then performed. Note that these three readings in a row are averaged to provide a baseline from which to judge any changes following pharmacology. These baseline recordings have been collated and analysed in Chapter 3.

The effect of two antagonists on evoked responses of WDR neurons were assessed:

- Ondansetron: A selective antagonist for the 5HT₃ receptor, with a selectivity ratio of over 1000 for this site compared to the other receptors.
- Atipamezole: A selective antagonist of the α_2 noradrenergic receptor, which shows a roughly >8000 fold selectivity for α_2 over α_1 , and greater potency than yohimbine.

where each animal only received one of these drugs. As such, of the four experimental groups of animals (Early MIA, Early Sham, Late MIA and Late Sham) half from each group received ondansetron, and half received atipamezole.

1) Ondansetron:

- Ondansetron (PLIVA Pharma Ltd) was purchased in vials of 2mg/ml solution.
- 50 μ l was administered directly to the spinal cord, using a Hamilton syringe, to provide a 100 μ g dose; this dose was selected based on previous work in the 2mg MIA model in which 100 μ g had provided successful modulation of evoked activity of single neurons in Lamina V (Rahman, Bauer et al. 2009).
- The effect of this dose was followed for an hour at 10, 30 and 50 minute intervals following administration, where the electrical, mechanical and thermal stimulation protocol used to characterize the cell was repeated at each of these time points.

2) Atipamezole:

- Atipamezole (Sigma, UK) was dissolved in 95:5 saline:DMSO to create solutions of 2mg/ml and 0.2mg/ml atipamezole.
- Doses were administered in a volume of 50 μ l, directly to the spinal cord, using a Hamilton syringe.
- The effect of two doses were followed in each animal, administered sequentially and spread over time such that:
 - Dose 1: 10 μ g applied directly after baseline characterization of each neuron (t=0).
 - Dose 2: 100 μ g applied at t=70 mins, such that the drug was being applied once all three rounds of characterization had been performed to assess the effect of dose 1, plus an additional 10minute buffer.

These doses were selected based on previous work in the lab where 10 and 100µg had provided successful modulation of evoked activity in Lamina V, including in a 2mg MIA model (Green, Lyons et al. 1998, Burnham 2012).

- The effect of each dose was followed for an hour after administration, at 10, 30 and 50 minutes, where the electrical, mechanical and thermal protocol used to characterize the cell's responses were repeated at each of these time points

4.2.5 Data Analysis

The effect of each drug is expressed as the maximum change in absolute terms from the baseline value per individual drug dose across all time points. All data is expressed as number of action potentials, presented as the mean \pm SEM.

Effect of ondansetron

- Naturally evoked (mechanical and thermal) responses: Difference between baseline and post-drug groups analysed using a two-way ANOVA followed by Bonferroni corrected post-hoc tests.
- Electrically evoked and dynamic brush responses: Difference between groups analysed using paired student's T test.

Effect of atipamezole

- Naturally-evoked (mechanical and thermal) responses: Difference between groups analysed using a two-way ANOVA followed by Bonferroni-corrected post-hoc tests.
- Electrically-evoked and dynamic brush responses: Difference between group (Baseline, 10 and 100 µg) analysed using one-way ANOVA with Dunnett's correction for multiple comparisons.

Values were deemed significant at $p < 0.05$.

4.3 Results

4.3.1 Effect of ondansetron in Early MIA animals

The effect of 100µg ondansetron on evoked responses of Lamina V WDR neurons was assessed in Arthritic and Sham rats 3-5days after injection of MIA or Saline respectively. No significant effect was observed in either group of animals, across electrical, mechanical and thermal stimulation.

No significant change in electrically evoked activity was observed in either Early MIA or Sham animals following application of ondansetron (Figure 4.1a). However, Early MIA animals exhibited a strong trend towards reduction in electrically evoked responses, notably Aδ ($p=0.051$) and windup ($p=0.054$).

No significant effect of ondansetron was observed in mechanically evoked responses, to either dynamic brush or punctate stimuli, in either group (2 way ANOVA)(Figure 4.1b, c & d). However, there is an observable trend towards inhibition of evoked mechanical responses, to both brush and punctate stimuli in the Early MIA animals that is not seen in the Sham groups. Similarly, though the effect of ondansetron was not significant for evoked responses to thermal stimulation in either group (2 way ANOVA) (Figure 4.1e & f), there is a sizable inhibition at 45°C in Early MIA animals, which if Bonferroni post tests were legitimate give a $p=0.081$, and at 40°C in Early Sham animals $p=0.020$. AUC analysis (data not shown) did not reveal any significant difference. Note also, in Figure 4.1b, that no difference of brush-evoked responses in early MIA animals vs. shams are observed, contrary to Figure 3.4b, and discussed in Section 3.4.3.

4.3.2 Effect of ondansetron in Late MIA animals

Ondansetron had no significant effect on Lamina V WDR neuron electrically evoked responses in Late MIA animals, but caused a significant inhibition of both the Aβ and Aδ counts in Late Shams animals (Paired T Test, * $p\leq 0.05$)(Figure 4.2a). This is a trend carried across the sham electrically evoked responses, with a reduction in post discharge nearing significance at $p=0.064$. This is similarly reflected in MIA Aδ counts in MIA animals following ondansetron.

No significant change in mechanically or thermally evoked responses following application of ondansetron was observed in either Late MIA or Late Sham animals (2way ANOVA, Figure 4.2c-f), though both groups exhibited a trend towards reduced punctate mechanically evoked activity. A similar trend was observed in thermally evoked responses in Late Shams, whereby Bonferroni post hoc tests indicated a significant reduction to 45°C water jet ($p=0.033$). AUC analysis (data not shown) did not reveal any significant difference.

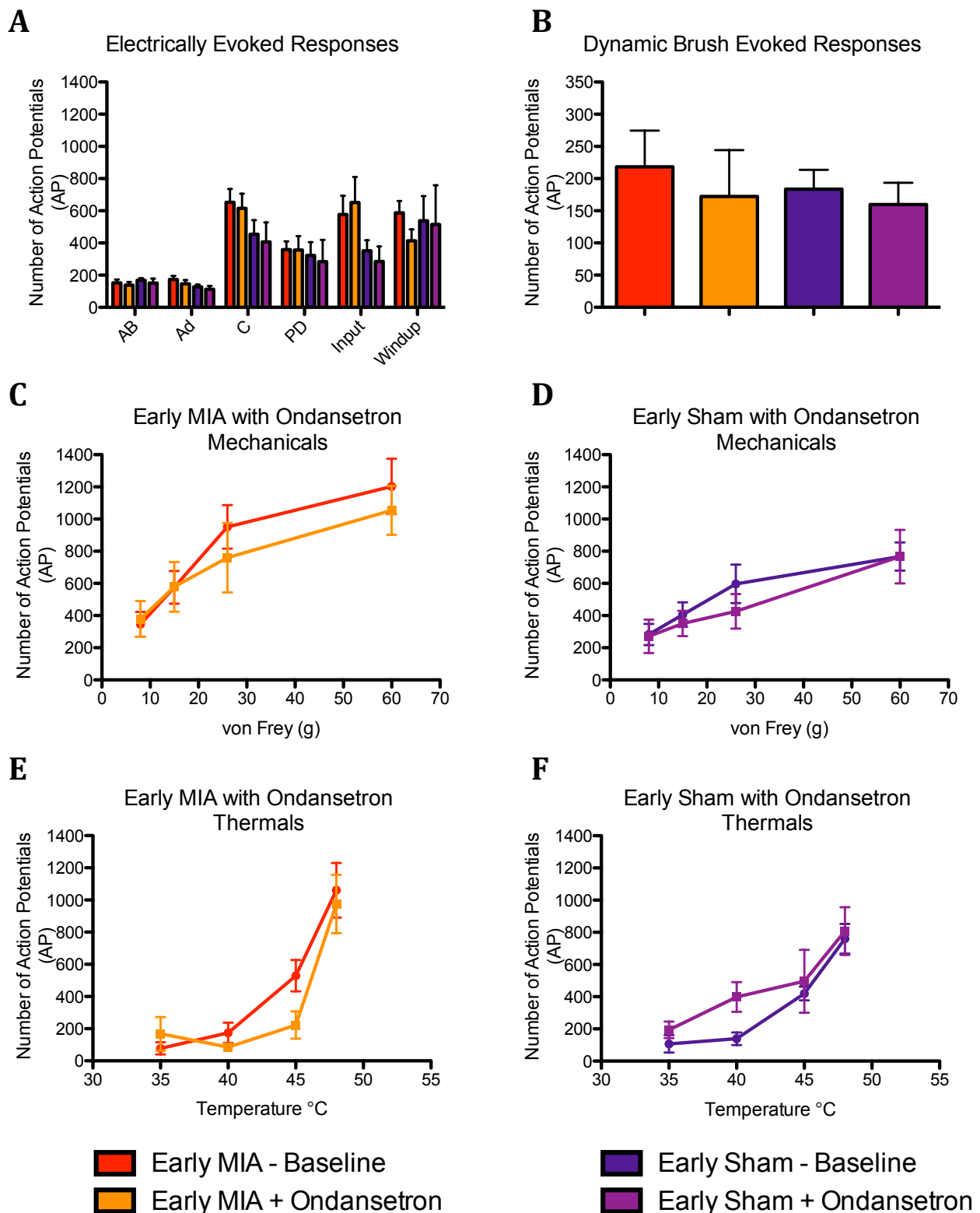


Figure 4.1 -100µg of spinally administered ondansetron has no significant effect on the evoked responses of Lamina V WDR neurons in Early MIA (n=7) or Early Sham (n=6) rats (Days 3-5 following injection). **A)** Electrically evoked responses are unaltered following ondansetron in both MIA and Sham **B)** Dynamic brush evoked responses are unaltered following ondansetron, though there is a trend indicating inhibition. Paired T-test assessed effect of ondansetron.

Second row: Punctate mechanical evoked responses in MIA (**C**) and Sham (**D**) are not significantly altered by the application of ondansetron, though a trend indicating inhibition of evoked responses is apparent to noxious mechanical stimulation in MIA animals. **Bottom Row:** Thermally evoked responses in MIA (**E**) and Sham (**F**) are not significantly altered by the application of ondansetron, however a notable trend towards inhibition of 45°C evoked responses is apparent in MIA animals to 40°C evoked responses in Sham animals. 2-way ANOVA assessed effect of ondansetron.

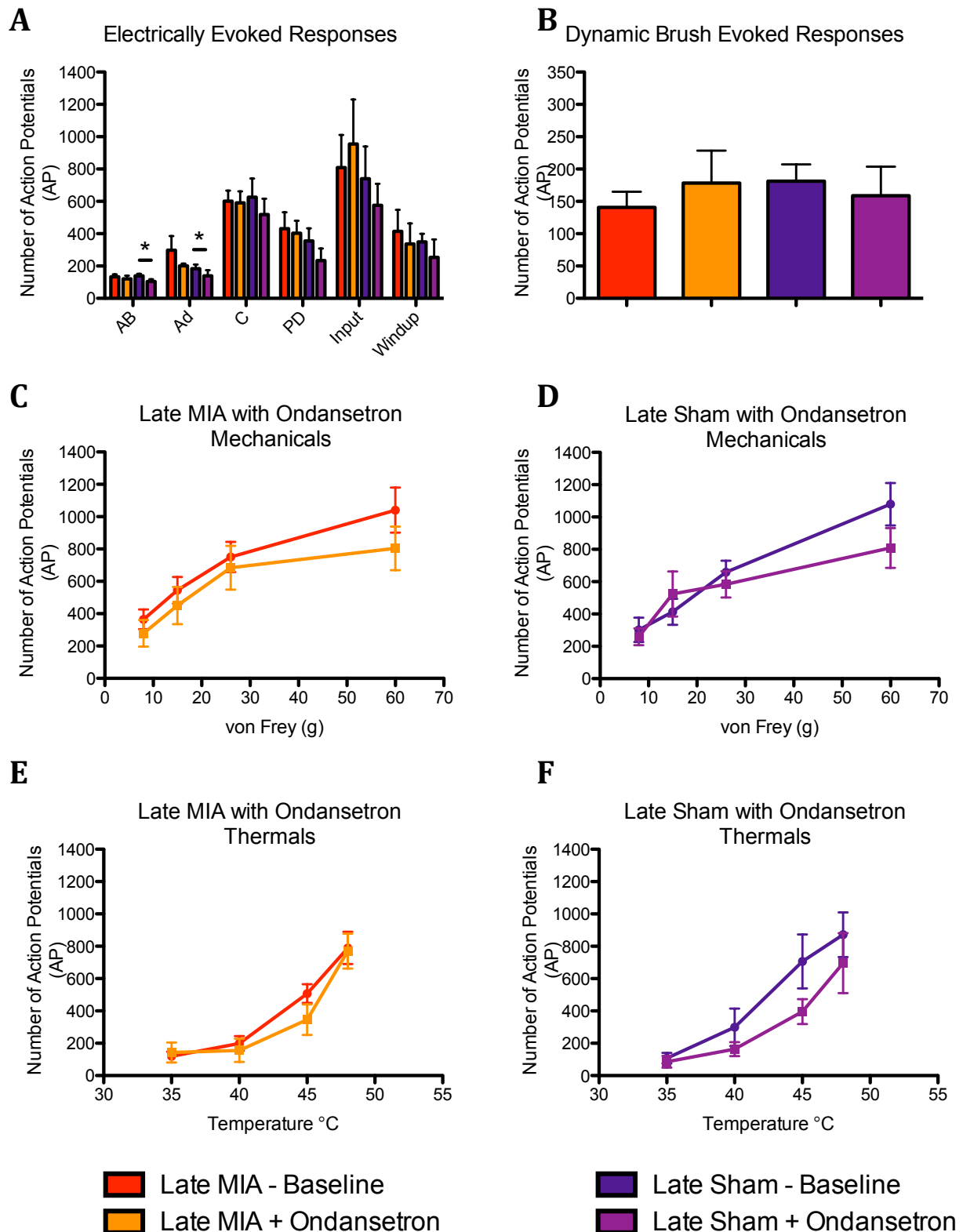


Figure 4.2 -100µg of spinally administered ondansetron has no significant effect on the mechanically or thermally evoked responses of Lamina V WDR neurons in Late MIA (n=6) or Late Sham (n=7) rats (Days 10-14 following injection), but significantly inhibited the electrically evoked Aβ and Aδ counts in sham animals. **A)** Electrically evoked responses are unaltered following ondansetron in MIA animals, but significant inhibition of Aβ and Aδ counts are observed in Shams. Paired T-test assessed effect of ondansetron (* p≤0.05) **B)** Dynamic brush evoked responses are unaltered following ondansetron in MIA and sham animals.

Second row: Punctate mechanical evoked responses in MIA (C) and Sham (D) are not significantly altered by the application of ondansetron. **Bottom Row:** Thermally evoked responses in MIA (E) and Sham (F) are not significantly altered by the application of ondansetron, however a notable trend towards inhibition of 45°C evoked responses is apparent in in Sham animals. 2 way ANOVA assessed effect of ondansetron.

4.3.3 Effect of atipamezole in Early MIA animals

The effect of sequential doses of 10µg and 100µg of atipamezole, when applied spinally, on the evoked responses of Lamina V WDR neurons was investigated in arthritic and sham animals, 3-5 days after the injection of either MIA or Saline respectively. Unlike previous work in the 2mg model, no significant effect was observed at either dose in either group for mechanical or thermally evoked activity (Figure 4.3).

One way ANOVA identified a significant effect of atipamezole on electrically evoked windup in Early MIA animals, in which Dunnett's multiple comparisons identified a significant reduction from 100µg of atipamezole (** $p \leq 0.01$). No significant effect was observed to any other electrically evoked measure in Early MIA animals, nor in Early Shams, as a result of atipamezole.

While One Way ANOVA and Two Way ANOVA failed to identify a significant effect of atipamezole in either dynamic brush or punctate mechanically evoked responses respectively in Early MIA animals, there is an unexpected trend towards an inhibitory effect of atipamezole. This effect nears significance for punctate mechanical evoked responses, at $p=0.082$, where the effects at vF26g following 100µg atipamezole or at vF60 following 10µg are clear. In contrast, no significant effect or trend is observed in the Early Sham animals, where responses closely aligned at all doses of atipamezole for dynamic brush and punctate mechanical stimulation. Note also, in Figure 4.3b that unpaired student's t test confirms the significant enhancement of brush-evoked responses in early MIA animals vs. shams (* $p \leq 0.05$), as observed in Figure 3.4b.

Atipamezole had no observable or significant effect on thermally evoked responses in either Early MIA or Early Sham animals.

4.3.4 Effect of atipamezole in Late MIA animals

Neither dose of 10µg nor 100µg of spinally applied atipamezole has a significant or observable effect on the evoked responses of Lamina V WDRs in arthritic or sham animals 10-14 days after the injection of MIA or saline respectively, across all three stimulus modalities. Evoked responses were highly consistent across the two doses in both animals groups to both mechanical and thermal stimulation. As in Early animals, there is arguably a trend towards reduced electrically evoked responses in late MIA animals, notably wind up.

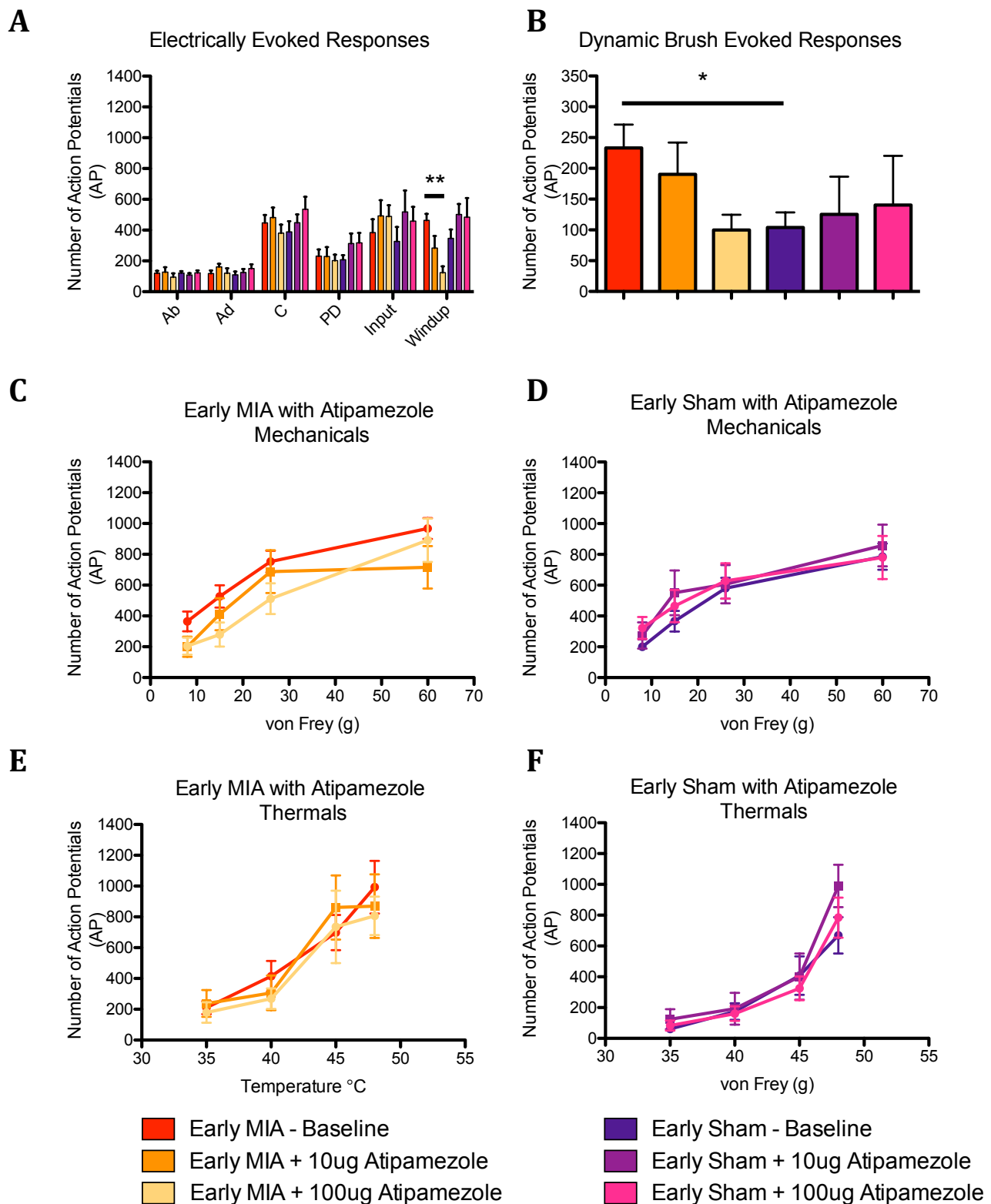


Figure 4.3 - Spinally administered atipamezole has no significant effect on the mechanically or thermally evoked responses of Lamina V WDR neurons in Early MIA (n=7) or Early Sham (n=7) rats (Days 3-5 following injection), but significantly inhibited the electrically evoked wind up in MIA animals. A) Electrically evoked responses are unaltered following atipamezole in Sham animals, but significant inhibition wind up is observed in MIA animals. A trend towards increased C fiber, PD, input and wind-up is apparent in the Sham animals. One-way ANOVA with Dunnetts multiple comparisons assessed effect of atipamezole (p<0.01) B) Dynamic brush evoked responses are not significantly altered by atipamezole in MIA and sham animals, though a trend towards inhibition is apparent in MIA animals. Responses are significantly enhanced in Early MIA vs. Sham (* p<0.05, unpaired student's T test)**

Second row: Punctate mechanical evoked responses in MIA (C) and Sham (D) are not significantly altered by the application of atipamezole. **Bottom Row:** Thermally evoked responses in MIA (E) and Sham (F) are not significantly altered by the application of atipamezole. 2 way ANOVA assessed effect of atipamezole.

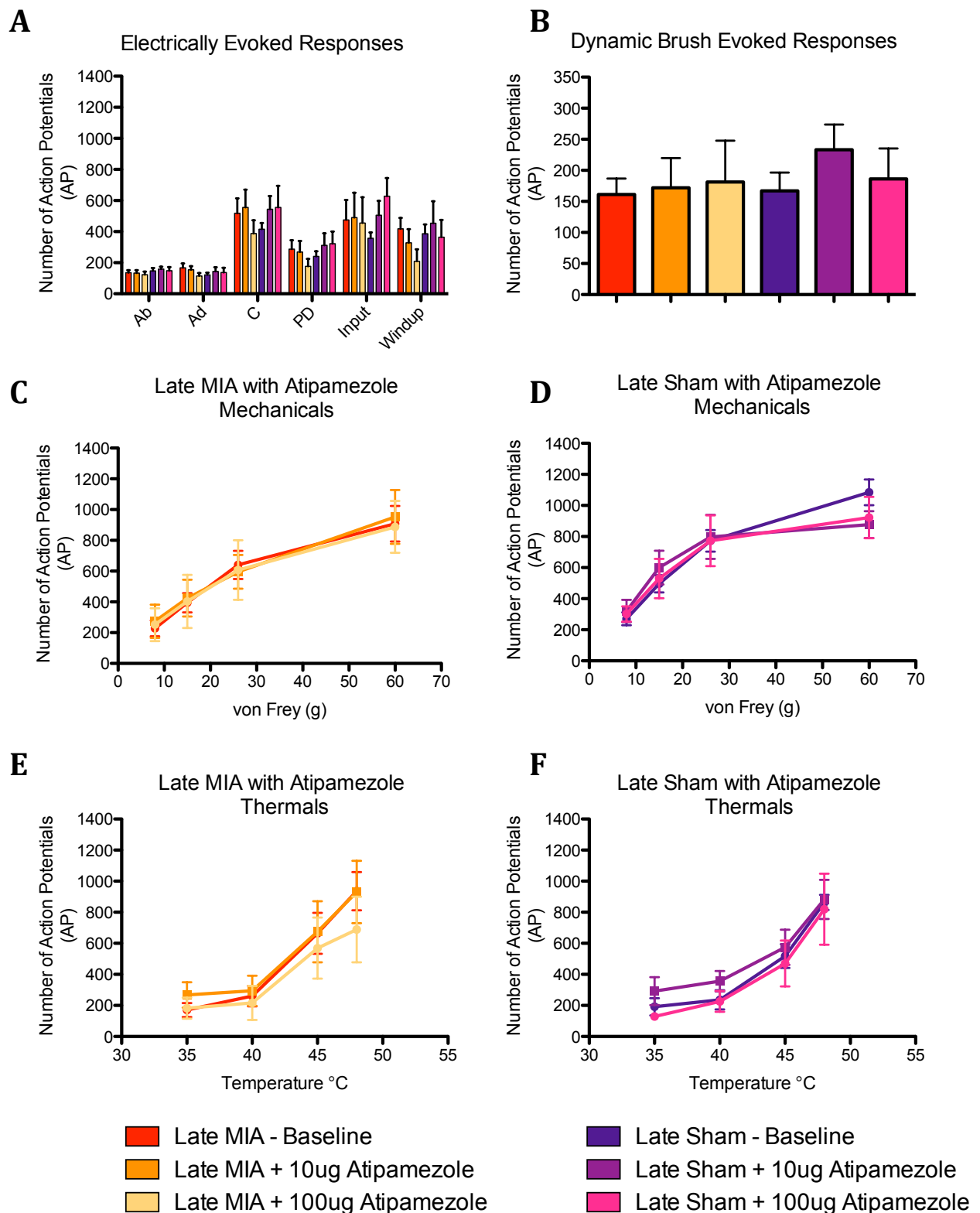


Figure 4.4 - Spinally administered atipamezole has no significant effect on evoked responses of Lamina V WDR neurons in Late MIA (n=7) or Late Sham (n=7) rats (Days 10-14 following injection) **A)** Electrically evoked responses are unaltered following atipamezole in both MIA and Sham. A trend towards increased C fiber, PD and input is apparent in the Sham animals, while a trend towards increasing wind-up is seen in MIA animals. **B)** Dynamic brush evoked responses are unaltered following atipamezole in both MIA and Sham. One-way ANOVA assessed effect of atipamezole.

Second row: Punctate mechanical evoked responses in MIA (**C**) and Sham (**D**) are unaltered by the application of atipamezole in both MIA and Sham. **Bottom Row:** Thermally evoked responses in MIA (**E**) and Sham (**F**) are not unaltered by the application of atipamezole in both MIA and Sham animals. 2 way ANOVA assessed effect of ondansetron.

4.4 Discussion

A role for adaptations in tonic descending controls in OA pain were first suggested by finding that, following spinalization in cats with acute inflammatory monoarthritis, deep dorsal horn neurons exhibited greater excitability and enhanced receptive fields, both at the knee and the paw (Schaible, Neugebauer et al. 1991). Subsequently, OA has been modeled using MIA injected into the joint, where selective antagonists have allowed the roles of individual transmitters and receptors to be teased out. These have pointed to time-sensitive adaptations in both the descending facilitatory and inhibitory systems on deep dorsal horn excitability in WDRs receiving inputs from the hindpaw (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). Herein I set out to establish if these adaptive changes are consistent in at a milder, non-neuropathic dose of MIA to model of OA pain. Findings here suggest they are not, adding further to our understanding that OA is a complex and highly plastic pain condition whose mechanisms and manifestations depend both on time point and extent of pathophysiology.

Herein I have demonstrated that at neither time point investigated in this 1mg MIA model is a significant effect of antagonism of either 5HT₃ or α_2 observed on the mechanically or thermally evoked responses of lamina V WDR cells, as in the sham groups, though trends may be apparent. This is in direct contradiction to findings in the 2mg MIA model, which have demonstrated significantly greater attenuation by ondansetron of thermal and non-noxious punctate and brush-evoked responses at days 14 (Rahman, Bauer et al. 2009), and significant facilitation by atipamezole of mechanically-evoked responses at days 3-5 (Burnham and Dickenson 2013), effects not observed in sham control animals.

4.4.1 The 1mg MIA model of OA fails to recruit descending serotonergic facilitatory systems

While plasticity of peripheral and spinal processes are crucial determinants of the pain experience, especially in chronic conditions, the contribution of descending serotonergic influences from the brainstem, which either facilitate, maintain or attenuate spinal excitability, have been demonstrated in inflammatory, neuropathic and visceral pain models (Urban, Zahn et al. 1999, Wei, Dubner et al. 1999, Green, Scarth et al. 2000, Terayama, Guan et al. 2000, Suzuki, Rahman et al. 2004, Bee and Dickenson 2007, Wei, Dubner et al. 2010, Sikandar, Bannister et al. 2012). While both these inhibitory and facilitatory systems may be active at once, it is postulated that it is the discreet balance between the two that may determine the profile of the pain experienced (Porreca, Ossipov et al. 2002, Vanegas and Schaible 2004).

Crucially, the net balance of inhibitory and facilitatory controls may differ between the primary and secondary pain sites, helping to explain the presence of referred pains – both in the clinic, where referred pain is correlated with the presence of brain stem sensitization (Gwilym, Keltner et al. 2009), but also in the MIA model, where changes in sensitivity are observed beyond the knee, in the paw. It has previously been demonstrated that local administration of lidocaine, or antagonists for NMDA or neurotensin receptors in the RVM attenuates the development of secondary thermal hyperalgesia during paw inflammation (Ren and Dubner 1996, Urban, Coutinho et al. 1999, Wei, Dubner et al. 1999). This points to a possible role for descending facilitations in driving, or at least maintaining, these secondary or referred pains. However, these studies focus on relatively acute periods of time, largely 3-5 days after the inflammatory insult. In the absence of work on descending serotonergic facilitation in the early days of the MIA model, these results predict a serotonergic drive of excitability of WDRs serving the paw in the early stages of the MIA model. Work from this lab has already pointed to increased drive acting at 5HT₃ at day 14+ of the 2mg MIA model, though it is of note that this does not necessarily mean that facilitation predominates, as descending inhibitory controls have also been shown to be increased or even dominant over facilitations at later stages of the model (Burnham and Dickenson 2013, Kelly, Dobson et al. 2013).

Electrically-evoked Responses

Previous work in this lab, across naïve, shams, SNL, 2mg MIA and carrageenan inflammatory models have failed to identify any effect of spinal application of ondansetron on the electrically evoked measures of spinal excitability in deep dorsal horn neurons (Green, Scarth et al. 2000, Suzuki, Rahman et al. 2004, Rahman, Bauer et al. 2009). This is broadly in line with findings reported here, in which no significant effect of ondansetron is observed in either early- or late-phase MIA animals, or early-phase sham animals. However, there are two notable exceptions – the evoked A β and A δ counts in late shams are significantly reduced following ondansetron, where A δ counts similarly show a strong trend towards reduction in early-phase and late-phase MIA animals ($p=0.051$ in early MIA).

If we first consider results presented in Chapter 3 (Figure 3.2), it can be observed that the A δ counts were enhanced in all MIA and the late-phase sham animals compared to early-phase sham animals. This relationship is significant for the comparison between Late MIA and Early Sham ($p=0.037$, unpaired T-test) and nears significance for Early Sham vs. Late Sham ($p=0.074$, unpaired T-test). Given conclusions from Chapter 3, that Late Sham animals may be exhibiting enhancements relating to long-term damage by injection process such that only early Shams represent the true control baseline, it could be postulated that in these three animal groups (Both MIA groups and Late Sham) there is a potentiation in

the range of the A δ fibers to electrical stimulation. Such changes have previously been observed in the 2mg MIA model(Vonsy 2008, Harvey and Dickenson 2009, Burnham and Dickenson 2013), as well as in studies considering the effects of capsaicin(O'Neill, Brock et al. 2012, O'Neill 2014). Such changes are broadly considered to be indicative of central sensitization, indicating the recruitment of A δ fibre input to WDRs, supporting the idea of central sensitization in both the early and late stages of the 1mg MIA model, and perhaps even the late sham animals.

If we then consider the effect of ondansetron, which reduces these A δ counts from electrically-evoked responses, it could be postulated that descending serotonergic systems may be facilitating the recruitment of these A δ fibers during MIA model. Because, with the exception of A β counts in late sham, no similar effect or trend is observed to the counts attributed to other fiber types or post discharge, it seems likely this could be a pre-synaptic effect which is predominantly restricted to A δ fibers, assisting in their recruitment and potentiation of electrically-evoked responses. This is in line with previous work characterizing the expression of 5HT₃ receptors in primary afferents, which identifies the predominant expression of these receptors to be on A δ fibers(Zeitz, Guy et al. 2002), as well as work identifying that PAG activation either facilitated or suppressed responses of dorsal horn neurons with A fiber or C fiber driven input respectively(Waters and Lumb 2008). (For an overview of the plasticity and functional significance of this see Heinricher et al 2009(Heinricher, Tavares et al. 2009)). It could be hypothesized that descending serotonin acts at 5HT₃ receptors on pre-synaptic terminals of A δ fibers to augment transmitter release and thus the likelihood of activating the secondary fiber. This sub-population of non-peptidergic and non-IB4 containing afferents with pre-synaptic 5HT₃ receptors are predominantly found in the superficial lamina(Kidd, Laporte et al. 1993, Miquel, Emerit et al. 2002, Zeitz, Guy et al. 2002), making it most likely that ondansetron restricts the passage of information by interneurons from A δ to WDR neurons in the deep dorsal horn.

As such, the apparent potentiation in the range of the A δ fibers to electrical stimulation in these groups following MIA and at days 10-14 following sham injection, plus the reduction apparent following ondansetron may indicate that the peripheral driver from the knee are activating descending serotonergic controls from the brainstem which facilitate the recruitment of A δ fibers during this state of central sensitization. However, this effect is only significant in late-phase shams and nears significance in early-stage MIA animals. It could be postulated that the contribution of serotonin to this process is relatively minor, thus explaining why this effect was not reported in previous studies – for example, in Green et al 2000 the application of ondansetron produced an average 19% reduction in A δ response(Green, Scarth et al. 2000); as all other electrically-evoked measures in this study remained at 99% of baseline values or above, this may represent a small but meaningful reduction in A δ fiber activity.

Mechanically-evoked responses

This study failed to identify any significant effect of the spinal application of 100µg ondansetron on either the dynamic brush- or punctate mechanically-evoked responses in either MIA or sham animals, at either time point. While a trend towards reduced evoked responses was observed, this lacks significance. As such, these results are far more in line with observations of the effect of spinal ondansetron in naïve and sham animals that do not show evidence of significant, active descending serotonergic facilitation of mechanically-evoked responses (Suzuki, Rahman et al. 2004, Rahman, Bauer et al. 2009). This is in direct contrast to findings in the 2mg MIA model, in which evoked responses to dynamic brush, vF2g and vF8g were reduced by more than 50% at this dose of ondansetron in MIA animals, but not shams (Rahman, Bauer et al. 2009). This work similarly showed no increase in 5HT₃ receptor mRNA in the DRG of MIA animals, which suggests these changes are the result of increasing transmitter as opposed to increased receptor expression driving facilitation.

Many reports describe how the facilitatory effects of descending serotonin contribute to excitability in the dorsal horn, as is also reflected in behaviour, including time spent licking or biting. The effects are seen in the second phase of the formalin response (Green, Scarth et al. 2000, Zeitz, Guy et al. 2002); in SNL and SCI models of neuropathic pain (Oatway, Chen et al. 2004, Suzuki, Rahman et al. 2004, Bee and Dickenson 2008); in models of visceral pain (Vera-Portocarrero, Yie et al. 2006, Sikandar, Bannister et al. 2012); and in carrageenan and CFA models of inflammatory pain (Rahman, Suzuki et al. 2004, Wei, Dubner et al. 2010). In contrast, blocking descending facilitatory pathways leaves baseline (naïve animal) electrophysiological and behavioural responses to mechanical stimuli unaltered – in line with this, 5HT₃ receptor knock out mice or mice with genetic deletion of 5HT₃ receptors show a normal acute pain profile (Zeitz, Guy et al. 2002, Kayser, Elfassi et al. 2007), similar to wild type mice, but the second phase of the formalin test is significantly reduced. This is in agreement with work utilizing molecular depletion of serotonin (Wei, Dubner et al. 2010), which showed unaltered acute pain profiles but reduced second phase formalin and WDR electrophysiology, which is unaltered by ondansetron in naïve and shams (Suzuki, Rahman et al. 2004, Rahman, Bauer et al. 2009).

As such, it is proposed that serotonergic facilitation is only activated by stimuli and conditions which generate a sufficient drive from the periphery, where descending facilitations contribute to the enhanced excitability of spinal cord neurons and the maintenance of a state of central sensitization (Bardin 2011). This barrage of nocifensive information thus triggers descending serotonergic facilitation, as shown by replication of the analgesic effect of selective ablation of NK1-containing ascending neurons in the superficial lamina on the second phase of formalin response by ondansetron (Suzuki, Morcuende et al. 2002). This is similarly suggested by Green et al 2000, who suggest a “state dependent action” of descending facilitation, and Peters et al 2010, who demonstrated

the failure of ondansetron to provide analgesic efficacy on PWT in SNL animals because of the failure of sub- or at-threshold stimuli to recruit descending serotonergic controls (Green, Scarth et al. 2000, Peters, Hayashida et al. 2010).

As such, the failure of ondansetron to significantly reduce the mechanically-evoked responses of either MIA or Sham rats, regardless of time point, in this 1mg model of MIA can be attributed to the severity, or lack thereof, of joint damage in this model. As discussed in Chapter 3, the 1mg model is established as a much milder OA model, both in terms of extent of inflammation, extent of pathological changes at earlier time points and lacking in neuropathic contributions. It could be interesting, given work of others in the 1mg model, which reveal a lack of behavioural sensitivity at day 14 but not day 28 (Kelly, Dobson et al. 2013), to investigate whether this remains the case at later days within this model – where it could be hypothesized that at later points, where bone pathology becomes far more pronounced, that descending facilitations could finally become engaged.

Thermally-evoked responses

In line with observations of the mechanically-evoked responses, no significant effect of ondansetron was observed in either MIA or sham animals, at either time point, on the responses of deep dorsal horn neurons following thermal water jet to the paw. As discussed above, this can be attributed to the inability of this milder model of OA to recruit descending facilitatory controls.

That serotonin contributes to secondary thermal hyperalgesia has been established in certain pain conditions. The molecular depletion of 5HT using shRNA for Tph-2 produced significant reductions in thermal hyperalgesia in both SNL and CFA treated animals, but failed to affect thermal paw withdrawal latencies in control animals. It is notable that these effects were time-dependent in the inflammatory model – indicating the plasticity of this descending facilitation during inflammatory pain (Wei, Dubner et al. 2010). Similarly RVM lesioning and spinalizations blocked the development of reduced thermal withdrawal thresholds at secondary sites (Urban, Jiang et al. 1996, Urban, Zahn et al. 1999, Urban and Gebhart 1999), supporting the importance of these controls in the development of thermal sensitivities.

Additionally, certain thermal stimuli may be considered sufficient to engage descending serotonin in naïve or sham animals, where strong engagement of A and C fibers may present a sufficient peripheral drive even in healthy animals. This goes some way to explain how ondansetron produces significant inhibition of thermally-evoked responses between 42-48°C in shams, SNLs and 2mg MIA animals but that the extent of inhibition is not significantly different between the control, acute and chronic pain groups (Rahman, Suzuki et al. 2004, Suzuki, Rahman et al. 2004, Rahman, Bauer et al. 2009). It similarly

explains how SP-SAP treated animals, which have NK-1 containing superficial neurons in the dorsal horn ablated, exhibit significantly reduced evoked responses to 42-48°C stimulation (Suzuki, Morcuende et al. 2002). This could account for the differences observed at 45°C in early-phase MIA and late-phase sham animals following ondansetron and 40°C in early-phase sham, which show near or significant reductions, when considering these temperature in isolation.

However, as previously mentioned, the 5HT₃ receptor has been shown to be unnecessary for acute pain responses to both 52.5°C hot plate, Hargreaves test and tail flick (Zeitz, Guy et al. 2002). This is not necessarily contradictory however. It may be that beyond certain highly noxious temperatures, certainly >50°C, the peripheral input from thermosensitive nociceptors is sufficient to maximally activate WDRs such that facilitation from 5HT₃ is no longer necessary nor relevant, such that this neuron cannot be further activated. This is not to say that serotonin is not still released in response to this stimulus, only that spinal WDRs are already responding maximally to a large peripheral input, so the impact of serotonin release is less relevant versus responses to lower temperatures.

Herein I have demonstrated that ondansetron has no significant effect on the thermally evoked responses of WDR neurones in a 1mg model of MIA, in direct contrast to previous results from a 2mg model. These results suggest that the 1mg MIA model is not severe enough to recruit descending facilitation, as is seen on other pain models. That some significant or near significant effect is observed in early MIA and sham animals in the middle of the thermal range may be attributed to the noxious drive of the temperature itself recruiting descending serotonin, an effect that loses physiological relevance at the upper end of the thermal test battery as the WDR approaches maximal rate of firing.

Overall, these results suggest that the 1mg MIA model of OA fails to recruit descending serotonergic facilitations of mechanical or thermally evoked responses at either days 3-5 or days 10-14, likely as a result of the milder profile of this smaller dose MIA model. This points to a mechanism in which descending serotonergic facilitation plays no part in the hypersensitivities observed at the paw in behavioural studies (Chapter 3, Figure 3.1). Given that it is believed by some that descending facilitations drive secondary but not primary hyperalgesia (Urban and Gebhart 1999), this also suggests that at these time points in a 1mg model of MIA that spinal neurons receiving inputs from the knee will similarly lack such controls.

While the wider implications of these findings to OA will be discussed alongside results of noradrenergic controls in *Section 4.3.1*, there are some issues specific to serotonergic regulation of the 1mg model that remain unanswered which could be addressed by future work. Namely – 1) Are facilitatory controls recruited at later time points in this 1mg model? 2) What role, if any, does serotonergic facilitation or inhibition have at the knee joint? 3) What role, if any, does descending

serotonin acting at 5HT₇ have in this 1mg model? The answers to these questions would perhaps provide a more complete picture of the role of the brainstem in enabling pain in OA.

4.4.2 The 1mg MIA model of OA fails to recruit descending noradrenergic inhibitory systems

Descending projections from the dorsolateral pontine (DLP) noradrenergic cell groups (most especially from the locus coeruleus [A6], A5 and A7 nuclei) provide a rich source of NA in the deeper laminae of the dorsal horn spinal cord (Westlund, Bowker et al. 1983, Westlund, Bowker et al. 1984, Clark and Proudfit 1991, Kwiat and Basbaum 1992). When these nuclei are activated they trigger the spinal release of NA which, through action at the α_2 receptor, provides an inhibition that curbs the excitability of spinal neurons and inhibits reflex responses in behavioural studies (Jones and Gebhart 1986). During states of central sensitization, especially in diseased states where inflammation drives a robust peripheral sensitization, these descending controls become enhanced to provide protection from both primary and secondary hyperalgesia (Tsuruoka and Willis 1996, Green, Lyons et al. 1998, Wei, Dubner et al. 1999, Tsuruoka, Maeda et al. 2004, Hughes, Hickey et al. 2013). It is suggested that these descending inhibitions prevent “response saturation” at the top end of the noxious stimulation scale and thus maintain accuracy of coding in the WDR neurons during periods of inflammation (Tsuruoka, Tamaki et al. 2012). As is the case for descending serotonergic facilitations, much of the evidence suggests that this pathway is not active during healthy, acute nociception (Hylden, Thomas et al. 1991, Tsuruoka and Willis 1996, Gutierrez, Nackley et al. 2003, Hayashida, Peters et al. 2012), but is instead activated by injury and inflammation. The contribution of this descending system in the 2mg MIA model of OA has previously been investigated in this lab, as outlined above, at both the early inflammatory and later nociceptive driven stage. This work revealed a α_2 driven inhibition of response of WDRs in early phase MIA animals to punctate stimuli, but had no effect in either late phase or naïve animals (Burnham and Dickenson 2013). Interestingly, the administration of atipamezole following milnacipran reversed deep WDR responses to both mechanical and thermal stimuli to pre-milnacipran baselines in early phase 2mg MIA and naïve animals, but failed to fully reverse this effect in late phase animals (Burnham and Dickenson 2013), suggesting a declining role for noradrenergic descending inhibition in the latter stages of this model – which may allow for a shift in the balance of descending controls to favour the facilitation observed at days 14 of the 2mg model (Rahman, Bauer et al. 2009).

In contrast to the results observed in this 2mg MIA model, no significant effect of 10 and 100 μ g atipamezole on the evoked responses of WDR neurons was observed at either the early or late stages of the 1mg MIA model, much as has previously been observed in sham and naïve animals. Importantly, given these doses of atipamezole have previously demonstrated efficacy, it seems less likely the absence

of effect observed here is dose related (Green, Lyons et al. 1998, Burnham and Dickenson 2013). An inference from these results may be that this 1mg MIA model is of insufficient severity to activate descending noradrenergic controls during these early stages of OA.

Absence of activation of descending noradrenergic controls

As previously mentioned, animals that do not exhibit pain states do not exhibit descending noradrenergic inhibition (Hylden, Thomas et al. 1991, Tsuruoka and Willis 1996, Gutierrez, Nackley et al. 2003, Hayashida, Peters et al. 2012). Put another way, naïve animals do not experience tonic noradrenergic regulation of spinal excitability. The results observed at both time points for the sham animals are in agreement with this, if we accept (contrary to some observed enhancements of baseline neuronal responses to stimuli in late stage sham animals) that these animals accurately model the absence of a pain state – as observed in behaviour in Chapter 3. While there is some evidence of tonic NA in the spinal cord, this is attributed to the role of NA in modulating ventral horn motor activity, where dorsal horn NA levels remain unchanged over time in naïve animals but show considerable increases during inflammation (Tsuruoka, Hitoto et al. 1999).

However, this does not necessarily mean that animals exhibiting a lack of noradrenergic descending inhibition do not have a pain condition. While activation of noradrenergic inhibition is intensity dependent, explaining much in the same way as with serotonergic controls how such inhibition could be absent in this low dose MIA, the changes are also dynamic over the time course of injury. Hughes et al 2013 suggest that this descending noradrenergic system spatially restricts and temporally delays the expression of neuropathic pain (Hughes, Hickey et al. 2013), but loses influence once neuropathic pain is established – as seen by the lack of an effect of α_2 adrenoceptor antagonism in neuropathic animals - using this argument to explain the lack of efficacy of NA-based therapies in neuropathic pain (Hughes, Hickey et al. 2013). The lack of α_2 adrenoceptor inhibition is similarly observed in electrophysiological studies with SNL animals, which exhibit clear behavioural hypersensitivity (Rahman, D'Mello et al. 2008). It can similarly be observed that antagonism at α_2 adrenoceptors in HZ rats that fail to develop neuropathic pain can “unveil” the pain state (De Felice, Sanoja et al. 2011), suggesting that the higher proportion (~85% vs. 50%) of SD rats which go on to develop allodynia in this model may experience the development of this chronic pain state due to the loss of noradrenergic protections. As such, there is clear evidence that not only can pain exist without the apparent engagement of descending noradrenergic systems, but seemingly it is the loss of these systems that may underlie the development of the chronic pain condition.

However, these examples relate to neuropathic pain while in the current work this 1mg dose of MIA has been explicitly chosen to avoid neuropathic contributions to pain, at both time points investigated. Such changes to NA controls have also been seen in inflammation: Danziger et al 2001 have shown in a CFA model of ankle arthritis that tonic descending inhibitions were enhanced during the acute stages (24 hours) and decreased over the chronic stages (3-4 weeks)(Danziger, Weil - Fugazza et al. 2001). They also argue that regardless of descending inhibition, spinal nociceptive outputs remain increased in both stages of inflammation due to increased peripheral input into the spinal cord.

Recent work from this lab has established that DNIC, the process by which noxious stimuli to one part of the body inhibits pain perception in another, has identified a clear role for descending NA at α_2 in the observed effects of DNIC, through both the use of atipamezole and Yohimbine (Bannister, Patel et al. 2015)). Given that a loss of DNIC is described in patients with OA(Arendt-Nielsen, Nie et al. 2010), the application of conclusions on the absence or loss of a NA pain protection system in these MIA animals may reflect the loss of DNIC in patients, where such changes may leave increases in spinal excitability and high outputs from peripheral barrage to the spinal cord unchecked, allowing for the development of referred pains observed in patients and rats.

In the above-discussed experimental protocols however, a descending NA 'protection' system has been engaged and then lost, for which there is no evidence in this investigation present work, since no atipamezole related enhancements in evoked activity are observed across either time point. In this 1mg MIA model, in contrast to findings in the 2mg model, atipamezole has no significant effect on evoked responses of WDR neurons at days 3-5 post-injection. This could be the consequence of one of, or a combination of, two possible factors. First, that in the 1mg model the size of the inflammatory insult, and thus the peripheral drive from the knee, is insufficient to engage tonic descending noradrenergic controls to this secondary site. Second, that this may relate to the differential regulation between primary and secondary sites.

Ren and Dubner, in their review of descending modulation in persistent pain, state that "This enhancement of descending inhibition appears to be present when the animal is subject to continuous, persistent noxious stimulation", where the primary afferent input is attributed to triggering this ascending-descending feedback circuit. However, unlike the serotonergic system which appears to require "sufficient" input above a certain threshold of nociceptive activity – given effects of 5HT₃ receptor antagonism are not seen in the carrageenan model (Green, Scarth et al. 2000)- the effects of descending NA are observed as early as 2 hours and as late as 5 days after insult across various "strengths" of inflammatory pain modeling(Tsuruoka and Willis 1996, Tsuruoka and Willis 1996, Green, Lyons et al. 1998, Tsuruoka, Hitoto et al. 1999, Gjerstad, Tjølsen et al. 2000). Such inflammatory pain models are likely to provide a rough equivalent to this early phase of the MIA model. As such, it seems

that pain sufficient to be observed in behavioural studies should be sufficient to activate descending NA controls.

We know from both the results presented in Chapter 3, of both changes in weight distribution and withdrawals from vF8g, and the characterization of behaviour in this model that animals exhibit pain-like behaviour at this time, at this dose(Thakur, Rahman et al. 2012). Perhaps then, during the electrophysiological recordings presented here, the lack of movement or weight bearing in the joint allowed a break from high-level activity from the knee, and thus immobility-related quieting of descending NA controls, while in the 2mg animal the insult to the knee is sufficient that without movement or weight bearing pressure there is still ongoing input to the spinal cord and DLP. While work from Kelly et al 2012 have demonstrated the presence of a significant spontaneous C-fiber drive from the periphery in immobile, anaesthetized 1mg MIA animals(Kelly, Dunham et al. 2012), this study used sodium pentobarbital. It has been shown previously that recordings from cortical neurons in the auditory center exhibit significantly reduced spontaneous activity under isoflurane vs. pentobarbital (Cheung, Nagarajan et al. 2001), and significant differences have previously been observed in the effects of isoflurane and pentobarbital anaesthesia in animals with inflammatory pains(Boegel, Gyulai et al. 2011) – though these are largely attributed to spinal GABA_A differences and may not be relevant to these peripheral recordings. To test this theory, it would be interesting to investigate the effect of joint movement upon noradrenergic control of spinal excitability – since previous work has shown that joint manipulation following capsaicin injection to the knee induces an α_2 driven antinociception(Skyba, Radhakrishnan et al. 2003).

As such, it is possible that the milder, 1mg model does not induce sufficient ongoing activity during rest to provide tonic, ongoing inhibition from descending noradrenaline – and instead this system may simply be recruited during noxious movement. The implication of this is two-fold. First, that in milder 1mg there is less ongoing pain and instead pain is movement evoked. Conditioned place preference (CPP) work across multiple MIA doses provides support for this hypothesis, as CPP following lidocaine to the affected knee is only observed in 3mg animals(Okun, Liu et al. 2012). Second, that SNRI therapies would only provide relief to movement evoked pains. It would be interesting to consider the effect of SNRI therapy on behavioural measures including Rota rod, night time movement and burrowing activities in the 1mg model to see if this is indeed the case.

How else can the lack of NA modulation be explained? It could be that these results can be attributed to the areas of stimulation on the animal. By testing evoked responses from paw stimulation, this study is exclusively considering a referred, secondary pain. It is suggested that during inflammatory pain that descending control systems discriminate between the sites of primary and secondary hyperalgesia, such that inhibitions predominate to the primary sites and facilitations predominate to the

secondary(Vanegas 2004). Indeed, many of the studies outlined above in which descending NA is engaged to limit spinal excitability and behaviour investigate the primary injury sites. Though, of course, the adaptations identified from previous work on 2mg MIA from this lab is focusing on a secondary areas(Burnham and Dickenson 2013). Perhaps, if work were to investigate NA controls to the knee in a 1mg model such controls would be evident, and may be an avenue for future investigation.

Ultimately, with the clear presence of hypersensitivity at this time point in these early-phase MIA animals, alongside work that well characterizes inflammation and pain in these animals, the absence of recruited descending noradrenergic inhibition from the LC is hard to explain. It is possible to attribute this to a bad batch of drug, however when this lack of effect was first observed a second, new batch of atipamezole was additionally dissolved and tested. This similarly failed to produce any effect.

The final (but very unlikely!) possibility, when considering the effect of atipamezole in these early MIA animals is that NA is in fact present in the dorsal horn, but instead facilitating excitability – given there appears to be a possible inhibition of evoked responses by atipamezole in the Early MIA animals, instead of the expected enhancements. While the lack of effect of atipamezole on evoked responses of WDRs in both MIA and Sham animals was relatively robust – with virtually indistinguishable response curves to vF and temperature in late MIA and Sham animals - there are two curious exceptions. First, the significant reduction in windup observed following atipamezole, and secondly, a trend towards *reduction* in mechanically evoked responses in the early MIA animals, though this is not statistically significant

How could atipamezole significantly inhibit windup in the early 1mg MIA model?

As can be seen in Figure 4.3a, increasing doses of atipamezole result in increasing reduction in wind up to evoked electrical responses in Early MIA animals, where the effect of 100µg was highly significant. The indication of such an effect is that, by some unclear mechanism, descending noradrenaline at days 3-5 is facilitating wind up such that its blockade results in a reduction to levels below those seen in sham animals. Traditionally the analgesic action of α_2 adrenoceptor activation is driven by the presynaptic inhibition of excitatory glutamatergic inputs from afferents, especially C fiber, and interneuron input(Sullivan, Dashwood et al. 1987, Kamisaki, Hamada et al. 1993, Pan, Li et al. 2002), as well as enhancing GABA release(Gassner, Ruscheweyh et al. 2009). Logically, for atipamezole to be inhibiting windup, NA would have to be enabling windup – perhaps by enhancing excitatory transmitter release from presynaptic button, possibly by reducing inhibitory transmitter release from inhibitory interneurons. In other words, the complete opposite to the actions previously characterized.

Other possibilities, that NA could be exerting an excitatory role through the inhibition of inhibitory systems or activation of excitatory systems are already established not to be the case by a wealth of previous study, reviewed by Pertovaara et al 2013(Pertovaara 2013), leaving these results as some what of an anomaly that I am unable to explain.

Does noradrenaline at the α_2 receptor facilitate mechanically evoked responses?

Similarly to the above, the spinal application of atipamezole in early MIA animals led to a trend towards inhibition of mechanically evoked responses. In Figure 4.3b there is a sizeable reduction in the evoked responses to brush observed in the 100 μ g of atipamezole test. Similarly, in Figure 4.3c the trend towards significant reduction to punctate mechanical evoked responses following atipamezole nears significance at $p=0.082$ and such that, if considered in isolation, the reduction observed to vF26g after 100 μ g atipamezole and the reduction at vF60g after 10 μ g would be significant.

This again presents the same issue discussed for wind-up – for atipamezole to provoke inhibition of evoked responses, NA must be facilitating mechanically evoked responses. As previously, control+saline responses exhibited in the appendix exclude flaws to technique underlying this decline in evoked activity. Similarly, the hitherto understood mechanisms of NA at α_2 in the spinal cord, and the effect of α_2 antagonism across a range of pain models, go against the observations in these early MIA animals.

4.4.3 Question of Central Sensitization in the MIA Model

In this chapter I have presented results that suggest that in this 1mg MIA model, at both the early and later stages, there is an apparent absence of a descending serotonergic drive or noradrenergic inhibition of spinal excitability in response to both mechanical and thermal stimulation. This aligns to results of Chapter 3, in which little to no significant enhancement of baseline evoked responses of WDR neurons in MIA animals are observed. This leads me to question the presence of central sensitization (CS), at least at the L5 segment serving the paw, during this 1mg model of OA at the time points considered, since WDRs are exhibiting neither enhanced evoked responses nor recruitment of descending controls. This instead suggests that, prior to day 14 of the 1mg MIA model, the pain observed in this model is likely driven by peripheral changes and strong nociceptive signals from knee pathology.

Though investigation of measures that may indicate CS in the 1mg model are less common, there are studies that refute my conclusion and point to a potential CS at these time points (See Table 4.1). Of 22

studies investigating pain in the 1mg MIA model of OA, 8 extend results indicative of a potential central sensitization in one of the time periods of interest. Notably however only one study, Sagar et al 2011, present data in which central sensitization at one of these time points is supported by multiple, diverse measures. Within this specific study treatment with 1mg MIA significantly decreased ipsilateral WB and PWTs of the hindpaw (distal allodynia), compared to saline-treated rats, from post-injection day 7 onwards. The numbers of Iba-1 positive, morphologically identified, activated microglia were also significantly increased in the ipsilateral spinal cord at days 7, 14 and 28 in MIA-treated rats vs. CL, and vs. Saline treated rats(Sagar, Burston et al. 2011). Additionally, oral administration of nimesulide (days 14-20) significantly attenuated MIA-induced decreases in weight bearing and distal allodynia. Such changes in the activation of microglia, combined with changes in distal allodynia, point to a CS from day 7 onwards (For discussion of microglia contributions to CS see(Woolf and Salter 2006)). However this study only found a significant correlation between the numbers of activated microglia in the ipsilateral spinal cord and distal allodynia at Day 28. While this advocates strongly for contribution of central sensitization to aberrant pain responses at these later stages, the failure to correlate the significant changes observed from day 7 onwards provides weaker support of an active contribution of central sensitization to the pain observed in the MIA model during days 10-14.

Of the remaining 7 studies whose results are suggestive of CS in either the early (3-5day) or late (10-14day) stages of the 1mg MIA model, all fail to conclusively refute my conclusions of the absence of CS in L5 lamina V at the time points studied (Table 4.1). In the case of Ivanavicus et al 2007(Ivanavicius, Ball et al. 2007) the observed efficacy of gabapentin at day 14 (the earliest time-point used for pharmacological assessment) may only be used confidently as surrogate indicator of central sensitization if the potential contributions of peripheral action are dismissed(Chapman, Suzuki et al. 1998, Hanesch, Pawlak et al. 2003). In the remaining 6 studies, evidence of CS relies upon the observed changes in PWT at these time points. As already discussed in Chapter 3 and this chapter, these behavioural hypersensitivities were observed in this work's own 1mg MIA rats at the early and later stages in behavioural studies without significant observable change to the evoked spinal cord electrophysiology of lamina V WDRs in late stage animals, or descending controls, suggesting such measures may be poor stand alone measure of CS. This is observed in Sagar et al 2010, one of these 6 studies, where hind paw withdrawal thresholds to mechanical punctuate stimulation were significantly decreased compared with those in rats receiving saline treatment, on days 3, 10, 14-28 - but no difference was observed between mechanically evoked WDR responses of MIA treated and sham rats on days 14-17(Sagar, Staniaszek et al. 2010). Meanwhile the study observed a significant increase in mechanically evoked responses at days 28- 31 in responses to 10 & 15g vF. Much like the studies presented in Chapter 3, this study failure to characterize evoked spinal responses at days 14-17 while demonstrating such changes at 4weeks post MIA.

Study	Time point	Measure (Pain specific)	Support CS in early?	Support CS in late?
Suggest CS at Both Time points				
Abaei et al 2016	5-32 days	WB, PWT, fMRI during IA Capsaicin (@28days +)	Yes*	Yes*
Sagar et al 2010	0-28 days	WB, PWT, SpEphys + CB1 or CB2 receptor antagonist / FAAH inhibitor	Yes*	Yes*
Suggest CS at Early (3-5 day) Time points Only				
Thakur et al 2012	3-19 days	Cooling, mechanical hypersensitivity, WB, IF, SpEphys + PGB	Yes*	No
Suggest CS at Late (10-14 day) Time points Only				
Burston et al 2013	0-28 days	WB, PWT, SpEphys, PCR, ELISA + CB2 receptor agonist	No	Yes*
Sagar et al 2011	7-28 days	WB, PWT, IF, IHC + nimesulide	No	Yes
Sagar et al 2015	0-24 days	WB, PWT, SpEphys + intra-articular NGF	No	Yes*
Ivanavicus et al 2007	8-35 days	WB, IF + GBP	No	Possibly
Mapp et al 2013	0-21 days	WB, PWT + Triamcinalone acetone	No	Yes*
1mg Studies That Do Not Support CS at These Time points				
Kelly et al 2012	3&14 days	WB, pEphys	No	No
Kelly et al 2013	3-28 days	WB, pEphys	No	No
Nagase et al 2012	0-28 days	Spontaneous night-time activity + NSAID /Gabapentin /Amitriptyline/ Opiates	No	No
Pomonis et al 2005	0-28 days	WB + morphine/ indomethacin/ celecoxib	No	No
Pulichino et al 2006	0-7 days	WB + IP antagonist	No	No
Rashid et al 2013	0 - 28 days	Tekscan® WB or pEphys+ dexamethasone /celecoxib / duloxetine /naproxen /morphine /pregabalin	No	No
Strassle et al 2010	1-21 days	WB + zoledronate	No	No
Yoshimi et al 2010	21+28 days	WB + AS1892802/ fasudil/ diclofenac/ tramadol	No	No
Burston et al 2016	0-27 days	WB, PWT + L-006235	<i>Unclear from abstract alone (OARS poster)</i>	
Bley et al 2006	Day 14	WB + rofecoxib/ RO1138452/ RO3244794	No	No
Bove et al 2003	1-14 days	WB + Paracetamol/ naproxen/ rofecoxib	No	No
Clements et al 2009	0-21 days	WB, IHC	No	No
Baragi et al 2009	0-14 days	WB, ELISA + MMP-13i	No	No
Cialdai et al 2009	0-14 days	WB, ELISA + MEN16132/ icatibant/ indomethacin	No	No

Table 4.5: Evidence for central sensitization in 22 published studies using the 1mg MIA model to investigate pain in OA. Those papers that did not involve the assessment of pain or related measures, such as electrophysiology, were excluded from the summary. The first 8 studies listed specifically noted changes consistent with or suggestive of central sensitization at the time points of interest, including mechanical hypersensitivity at the paw or changes in immune-fluorescence or pharmacological parameters, where * indicated those studies observing a change in PWT without investigation of or significant change in additional parameters at the time points of interest. The remaining 14 studies either characterized later time points, engaged measures unable to characterize central sensitization, most commonly WB, or the resource was unavailable online to read in full. (WB = weight bearing asymmetry, PWT = hindpaw mechanical withdrawal thresholds, IHC = Immunohistochemistry, IF = immunofluorescence, SpEphys = Spinal Electrophysiology, PEphys = Peripheral Electrophysiology) (Bove 2003, Pomonis, Boulet et al. 2005, Bley, Bhattacharya et al. 2006, Pulichino, Rowland et al. 2006, Ivanavicius, Ball et al. 2007, Baragi, Becher et al. 2009, Cialdai, Giuliani et al. 2009, Clements, Ball et al. 2009, Sagar, Staniaszek et al. 2010, Strassle, Mark et al. 2010, Yoshimi, Yamamoto et al. 2010, Sagar, Burston et al. 2011, Kelly, Dunham et al. 2012, Nagase, Kumakura et al. 2012, Rashid, Theberge et al. 2012, Thakur, Rahman et al. 2012, Burston, Sagar et al. 2013, Kelly, Dobson et al. 2013, Mapp, Sagar et al. 2013, Rashid, Theberge et al. 2013, Sagar, Nwosu et al. 2015, Abaei, Sagar et al. 2016, Burston, Xu et al. 2016)

In the remaining 14 1mg MIA studies, many presented evidence supporting CS at later time points but the study methodology had not allowed for assessment during the earlier stages, while others had used WB as the sole behavioural measure of pain and thus failed to capture any potential sensitization in uninjured tissue such as the paw. One study could not provide evidence as the abstract refereed to a poster unavailable for review online.

As such, it seems apparent that evidence supporting potential CS in these earlier stages of the 1mg model is inconclusive. It may be that at later stages, perhaps days 21-28, central changes in this smaller dose model of OA pain may become apparent or dominant, including the recruitment of descending controls, given the “unmasking” effect spinalization has been shown to have at 28th day of this model(Kelly, Dobson et al. 2013), the role of activated microglia in distal allodynia(Sagar, Burston et al. 2011), and the efficacy of selective NA re-uptake inhibition to resolve incapacitance at week 3 of the 1mg MIA model(Whiteside, Dwyer et al. 2010).

It would be interesting to consider what evidence of CS spinal recordings at higher lumbar levels might detect, notably in spinal segments with greater knee input versus L5, where this work records. Conclusions about the presence of central sensitization at those higher levels, largely levels L3 and L4 (with overlapping input with the paw into L5(Edoff, Grenegard et al. 2000)) cannot be drawn from this thesis’ data. To gain a more complete picture of the dynamic and seemingly knee pathology dependent changes in the descending controls it would be beneficial to characterize the effect of monoamine antagonism on WDR evoked responses serving the knee joint itself.

Clearly, the conclusions drawn here, pointing to the absence of descending control, also have implications upon the mechanisms by which pain might be referred to the paw, given the behaviour observed in Chapter 3. This favours the theory of receptive field expansion of secondary hyperalgesia, in which sensitization of ascending fibers serving the knee lower the threshold of activation such that previously weak or silent inputs from neighbouring regions (like the paw) become sufficient to activate ascending neurons, as discussed by Schiabe and Richter 2004. Interestingly, we could consider whether these changes are occurring at a higher spinal segments, or in a different lamina. Such changes in ascending lamina I NK1 positive neurons have been characterized in rats following CFA inflammation, seen as both the increase in receptive field(Hylden, Nahin et al. 1989) and shift to a greater proportion of monosynaptic A δ inputs(Torsney 2011). Most interestingly, receptive field expansions have previously been characterized in a 2mg model of MIA in lamina I but not lamina V ascending neurones during evoked spinal cord electrophysiology(Thakur 2012) – a study which similarly demonstrating that lamina I but not Lamina V neurones at day 14 post 2mg MIA had potentiated responses from fibers in the A δ range. Such changes could not have been observed in the present lamina V study. As such, the potential expansion the receptive field of lamina I ascending

neurons, such that paw inputs are also transmitted and interpreted as noxious, might explain the observed changes in behaviour in this 1mg MIA model.

4.4.4 Study Limitations

In considering the results and potential conclusions of this chapter, it is important to consider the limitations of the present study's design – both to provide context that will inform the conclusions, but also suggest potential modifications to the study that would have allowed greater clarity.

Dose Selection and Pharmacology

Within this study, single or two-dose pharmacology was performed using doses of the selected antagonist previously characterized as exerting an effect in the 2mg MIA model by Rahman et al 2009 or Burnham et al 2013 for Ondansetron and Atipamezole respectively (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). However, the assumption that a dose sufficient to alter electrophysiology in the 2mg model cannot be made with the same confidence that multiple ascending doses could provide.

As already discussed, it has previously been observed that spinal application of either ondansetron or atipamezole has no significant effect in naïve or sham animals at doses that affected MIA or peripheral nerve injury (Suzuki, Rahman et al. 2004, Rahman, Bauer et al. 2009, Burnham 2012). This is similarly the case in Sham animals in Figures 4.1-4.4. While this supports the concept of recruited descending control in OA and neuropathic pain models, it does not exclude the possibility of descending serotonin or noradrenaline completely. The sensitivities of the animals to antagonism may simply differ, for a variety of reasons – including receptor population, transmitter release etc.

In this instance, the use of multiple ascending doses would have allowed the construction of a dose response curve for both MIA and sham animals. This would have provided greater confidence in the ultimate absence of descending serotonergic or noradrenergic modulation, and potentially greater clarity on the differing extent of descending modulation of variously evoked neuronal responses between the 1mg and sham animals.

n Number and Power Equations

Conscientious animal research requires experimenters to find the balance between sufficiently powered experiments that do not miss significant differences and the use of the minimum number of animals required, to avoid unnecessary wastage of animal lives. In the execution of these experiments, I chose to finish with an $n=7$ per group as it was becoming clear that ondansetron and atipamezole were having no observable separation from baseline electrophysiological profiles. This was driven by a desire to limit the number of animals used, which totaled 54 rats without consideration of those rats in which experiments failed (e.g. premature death, inability to find and characterize a cell), and an awareness that n numbers of 7 or 8 had successfully characterized significant effects in the 2mg MIA studies from which my work followed (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013).

With hindsight, I should have performed power calculations to define the minimum number of animals, in line with ARRIVE guidelines (Kilkenny, Browne et al. 2010, Charan and Kantharia 2013). This would have defined an objective, as opposed to subjective, cut off. That said, performing power calculations using the data from Rahman et al 2013 suggests a sample size between 6-10. All data used was from the same dose of ondansetron used in this study, where the sample size suggestion depending on the evoked response selected to estimated group means and standard deviation. It is possible that if I had continued with the experiments to the upper bracket of this range, using a total of 80 rats, I might have seen a significant effect of ondansetron or atipamezole not detected at the current study power.

Evidence of Structural Pathology

Another major limitation in the discussion of the results presented in this chapter, as in all the chapters of this thesis, is the absence of analysis of the structural pathology of these animals. While previous literature has established the histopathological changes associated with both the early (Strassle, Mark et al. 2010, Kelly, Dunham et al. 2012) and late (Kelly, Dunham et al. 2012, Thakur 2012) stages of a 1mg MIA model, it would be a fallacy to assume that every injection of MIA was successfully delivered. Even in a clinical setting, IA therapies commonly miss their mark (in much larger knees) without ultrasound guidance (Berkoff, Miller et al. 2012). There are similarly considerations of the potential off-target effects of MIA, such as leaking from the synovial joint to affect surrounding tissues and drive pain independently of OA-like pathology – including potential uptake by local neuronal endings to trigger neuropathic pain. These possibilities undermine the assumption that a pain profile necessarily demonstrates a successful injection and the presence of OA-like changes in the knee joint.

Such variability of success of injections might also explain the inconsistency of the presence of enhanced evoked responses to dynamic brush observed in the baseline responses of the ondansetron treated and atipamezole treated cohorts of early 1mg MIA and sham rats (See section 3.4.3). An understanding of the differing structural pathophysiology or off target effects due to failed injections could provide clarity of potential mechanisms underlying this difference that the currently presented data alone cannot.

The conclusions drawn in this study (Chapters 3 and 4) are based upon potential considerations of the extent of histopathological changes – inflammatory in the early animals, and structural in the late animals. These conclusions would be far stronger with a) evidence of such changes, on which to base these conclusions and b) a correlation analysis, to detect any potential relationship between the extent of structural pathology and behaviour, electrophysiology, or pharmacological outcomes. Of note, previous analysis of this kind has failed to characterize a relationship between structural pathology and nociceptive behaviour or electrophysiology (McDougall, Andruski et al. 2009, Kelly, Dunham et al. 2012), suggesting alternate explanations for the differences observed between this work and earlier studies in the 2mg MIA would be required.

Such analysis could also allow the exclusion from analysis of animals in which OA had not been successfully induced by the MIA model. It is possible that in so doing, more significant differences between groups and after pharmacology might be detected. Conversely, in a meta analysis of studies that measured behavioural pain outcomes in small animal models of OA, Suokas et al 2014 demonstrate that *“Lack of reported evidence that OA structural change was successfully induced in the model was strongly associated with larger effect sizes”*, where effect size refers to reported analgesic efficacy (Suokas, Sagar et al. 2014). This analysis suggests that incomplete phenotyping of animals, including the failure to confirm structural pathology, prior to pharmacological interventions may lead to false conclusions. In this instance, the false conclusion would be to attribute differences in the response to ondansetron or atipamezole in these 1mg animals vs. previous 2mg studies to structural pathological differences, when these differences may instead related to off target effects of MIA in tissues surrounding the joint capsule.

Direct Comparisons Between 1mg and 2mg Animals

As discussed with n number limitations, the desire not to waste animal life must be balanced by gathering sufficient information for confident conclusions. In opting not to include a 2mg MIA cohort within these studies, I had sought not to replicate work already published and to instead use it as a comparator reference (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). However, given the

previously discussed variability and lack of consensus on whether the MIA model results in increased evoked responses to mechanical and thermal stimulation, and the absence of structural pathology analysis in these reference studies(Rahman, Bauer et al. 2009, Burnham and Dickenson 2013)., the analysis of results in this study are similarly limited by the absence of a 2mg MIA cohort for comparison. Though this would have required the repetition of previous studies, it would have facilitated direct comparison between sham, 1mg and 2mg MIA animals without accounting for inter-experimenter variability and in conjunction with joint histopathology could have allowed analysis of any potential relationship between joint pathology and evoked electrophysiology and pharmacology.

4.4.5 Overall Implications

Overall, these results might suggest additional considerations during the selection of pharmacotherapy for the management of OA pain. These results suggest that in mild, non-neuropathic OA, where the extent of pathological changes are still relatively limited and the pain less severe, that patients might not benefit from tricyclic's or SNRIs which manipulate these descending control systems. As is already the case in patient treatment pathways, and as has been validated in the MIA model, these patients may best benefit from therapies which focus on the peripheral origins of pain – notably NSIADs and paracetamol, where patients may only benefit from progression to antidepressant therapies once their pain becomes more pronounced and engages brainstem controls.

Chapter 5 – Brainstem Sensitization in Osteoarthritis Pain

5.1 Introduction

The state of central sensitization (CS), characterized by reduced threshold, increased responsiveness and enlargement of the receptive fields, is a term that describes changes in the spinal cord during pain (Cook, Woolf et al. 1987, Hylden, Nahin et al. 1989, Woolf 2011). Such changes are held to underlie hyperalgesia and allodynia during chronic pain states, and crucially to the OA pain experience – the referral of pain to healthy, unaffected tissue (Skagerberg and Björklund 1985, Giamberardino 2003). However, what is becoming increasingly clear is a picture in which CS is either maintained or limited by the presence of descending controls (Suzuki, Rygh et al. 2004). Similarly, chronic pain not only causes adaptive changes in spinal processing but drives plasticity of synaptic efficacy and patterns of cell activity in the RVM (Vasko, Pang et al. 1984, Fields, Heinricher et al. 1991, Potrebic, Fields et al. 1994, Gao and Mason 2000, Terayama, Guan et al. 2000, Cleary and Heinricher 2013), part of a process which has previously been coined as brainstem sensitization. By better characterizing these adaptations we may better understand the drivers underlying OA pain.

5.1.1 Heterogeneity of RVM Neurones

The RVM comprises one half of a critical pain control axis in the brainstem. In communication with the PAG, and integrating inputs both from the spinal cord and surrounding nuclei, the RVM exerts a bilateral descending facilitatory and inhibitory control over synaptic strength in the spinal cord such that it modulates nociceptive processing. It has become apparent that the RVM may both enhance and diminish nociceptive processing, ultimately determining the extent of pain perception. This is based in large part on the distinct populations of cells in the RVM which are recruited, where the distinct roles of ON-, OFF- and Neutral cells, defined by their distinct firing patterns, has become increasingly apparent. Early studies defined the ON cell as those which increase their firing just prior to the initiation of nociceptive reflex (such as tail flick in the rat) while OFF cells are tonically active and decrease firing prior to nociceptive reflexes. Neutral cells show no real change in activity prior to or during the nociceptive reflex (Fields, Bry et al. 1983, Heinricher, Barbaro et al. 1989).

The roles of these sub populations, most especially their patterns of firing, in relation to nociception and analgesia has become increasingly apparent. During periods of increased OFF cell activity there is an observable increase in latency to tail flick and conversely increased ON

cell activity shortens this latency (Barbaro, Heinricher et al. 1989, Heinricher, Barbaro et al. 1989). As such, it makes sense that a pharmacological agent that increase OFF cell activity and decrease ON cell activity would prove successful analgesics – exactly the mechanism underlying opiate analgesia. The administration of morphine causes significant reduction in the firing of ON cells while enhancing the activity of OFF cells(Heinricher, Morgan et al. 1994). It has been shown that the systemic or iontophoretic administration of morphine reduces both the spontaneous and nocifensive reflex related firing of ON-cells at doses which inhibit noxious heat related tail flick(Barbaro, Heinricher et al. 1986, Heinricher, Morgan et al. 1992). Conversely, systemic administration results in ongoing activity from OFF-cells following morphine(Fields, Vanegas et al. 1983, Heinricher, Morgan et al. 1992). During normal/acute pain states this activation of OFF cells is both necessary and sufficient for opiate analgesia, while inhibition of ON-cells alone is insufficient (Fields, Basbaum; et al. 2006). However, during chronic pain states such as inflammation the direct opioid inhibition of ON-cells has a much greater impact on hyperalgesia (Porreca, Ossipov et al. 2002), a result of the increased activity of ON-cells during inflammatory pain. While opiates produce direct inhibition of ON-cells, through μ -opioid receptors(Heinricher, Morgan et al. 1994, Marinelli, Vaughan et al. 2002), the action on OFF-cells is the result of dis-inhibition(Heinricher, Morgan et al. 1994), where the inputs of the opioids targeted inhibitory GABAergic neurones likely originate from beyond the RVM. This allows independent functional changes in these two populations(Cleary, Neubert et al. 2008).

The heterogeneity in function similarly extends to the effects of excitatory neurotransmitters - where small doses of Glutamate produces descending facilitation to the dorsal horn, while high dose Glutamate inhibits transmission (Zhuo and Gebhart 1992, Zhuo and Gebhart 1997). These differences seemingly result from the differing roles of different Glutamate receptors on ON and OFF cells. NMDA receptor antagonism by AP5 attenuates or blocks OFF cell activation/disinhibition, but had no effect on ON cell dischargers, while the AMPA and Kainate receptor antagonist CNQX significantly attenuated this ON cell nociceptive reflex related discharge(Heinricher, Schouten et al. 2001). This study similarly highlighted the role of NMDA recruitment to the analgesic effect of Morphine, through the NMDA mediated activation/disinhibition of OFF cells.

Their transmitter content can similarly distinguish these neurons. It is postulated that some 40% of RVM neurones projecting to the spinal cord contain serotonin (Marinelli, Vaughan et al. 2002). As such, these serotonergic cells may themselves be in the minority within the RVM(Skagerberg and Björklund 1985), of which some, but not all, are responsive to opiates - roughly 33% to μ and 25% to κ agonists. The remaining 60% of spinally projecting RVM

neurones may be GABAergic, of which 67% were μ responsive (Marinelli, Vaughan et al. 2002). In other words, those neurones projecting to the dorsal horn can be subdivided by serotonergic and GABAergic content.

It had been suggested that serotonergic neurones represent an independent population, composed of neither ON nor OFF cells (Potrebic, Fields et al. 1994, Gao and Mason 2000). This is contradicted however by both the identification of 5HT containing ON cells, designated by the expression of the μ opioid receptor (so called MOR+) (Marinelli, Vaughan et al. 2002), and the ability of 5HT depletion by the neurotoxin 5,7-dihydroxytryptamine to block the analgesic effect of morphine microinjection into the RVM, believed to rely on OFF cells (Vasko, Pang et al. 1984, Fields, Heinricher et al. 1991). Crucially ON and OFF cells are not believed to have one single neurochemical designation, though both may have subsets of 5HT containing neurones. It has additionally been proposed that perhaps those serotonergic cells, which are neither ON nor OFF cells, are in fact neutral cells whose characteristics adapt during pain conditions to underlie the plasticity of descending controls during pain conditions (Ellrich, Ulucan et al. 2000, Miki, Zhou et al. 2002).

In addition to 5HT, OFF cells drive direct inhibition through GABA – as evidenced by most OFF cells staining for GAD, the GABA synthesizing enzyme (Winkler, Hermes et al. 2006). However, ON cells similarly stain for GAD – an observation which may seem hard to resolve with the nociceptive facilitatory role of this cell population (Mason 2012). This could be attributed to a possible inhibitory effect of ON cells on OFF cells (Fields, Heinricher et al. 1991).

Meanwhile the picture for descending facilitation is not yet fully understood. Lesioning studies suggest that descending facilitation is driven by two distinguishable groups of neurones, those defined by 5HT content and those defined by μ opioid receptor expression, considered to represent the ON cell population. Crucially, when comparing the effects of 5HT or MOR+ cell depletion, it is observed that while both attenuate the development of inflammatory pain the effect of MOR+ depletion is more prolonged, likely due to the overlap between these two cell populations (Carr, Géranton et al. 2014). This study similarly went on to suggest a possible mechanism of non-serotonergic facilitations, likely through the positive modulation of the immune cell process in the dorsal horn by MOR+ cells, identifying a role for iNOS and chemokines including CXCL9 and CXCL10 in dorsal horn excitability (Carr, Géranton et al. 2014).

As such it is clear that subdivisions of cells in the RVM is far from simple, though I have tried to simplify this in Figure 5.1.

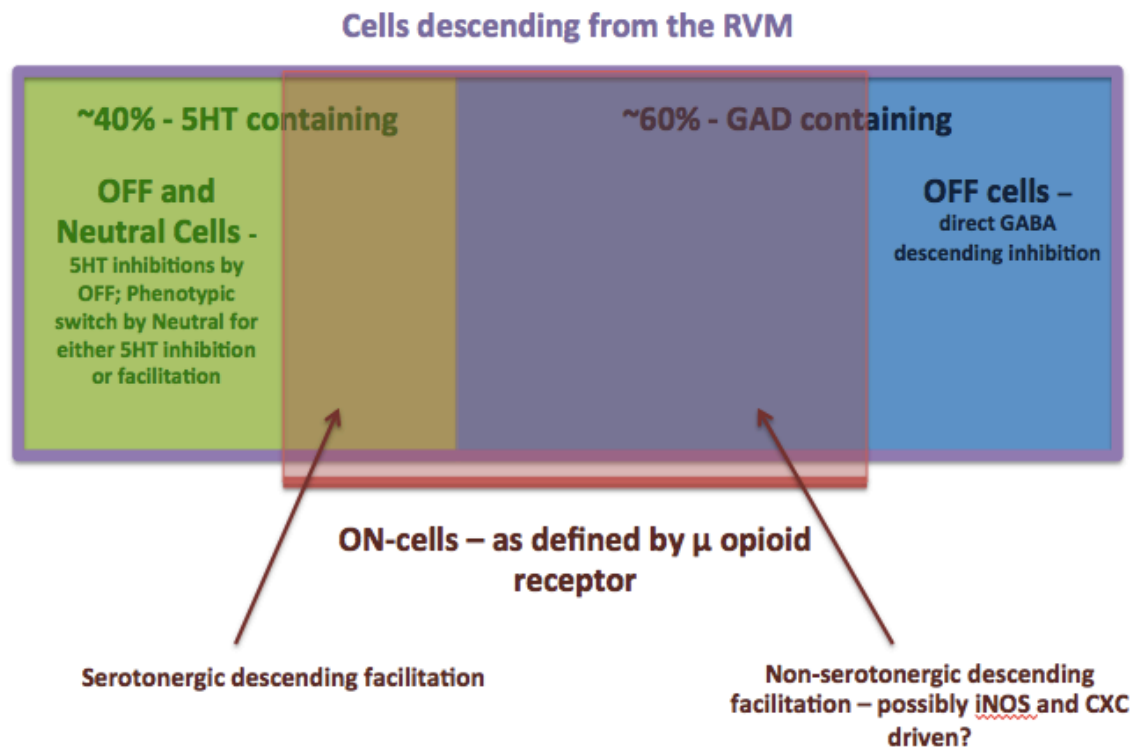


Figure 5.1 – Pictorial summary of a simplified possible sub-classifications of neurons descending from the RVM, based upon Barbaro 1986.; Heinricher 1992; Fields, H., et al 1983; Porreca 2002; Cleary 2008; Zhuo and Gebhart 1997; Zhuo and Gebhart 1992; Heinricher 2001; Winkler et al.2006.

5.1.2 Evidence of Brainstem Plasticity During Pain

It has been observed, through various combinations of lesioning, neurotransmitter depletion, pharmacology studies, electrical stimulation (ES) and PCR, that the RVM is highly plastic and changeable during the time course of a pain condition, be it inflammatory or neuropathic. Here I will specifically consider inflammation.

During the initiation period, namely the first 3 hours, RVM controls shift to favour descending facilitation of dorsal horn processing (Terayama, Guan et al. 2000), driven by plasticity of the NMDA receptor population (Terayama, Dubner et al. 2002). It was similarly shown that molecular depletion of 5HT from the NGC α neurons attenuates the development of mechanical hyperalgesia and allodynia after CFA injection, suggesting serotonergic neurons have a significant role in facilitating the development of hyperalgesia following inflammation (Wei, Dubner et al. 2010). This differs considerably from neuropathy, where it has been observed that descending facilitation is not a requirement for the initiation of pain in neuropathy, as it is for inflammatory pain, but is a requirement for its maintenance (Burgess, Gardell et al. 2002).

Given this information, it seems feasible that in the initial hours of inflammation the sensory barrage ascending to the brain stem activates facilitatory descending controls. As with peripheral sensitization, this early facilitation is beneficial as a mechanism for limiting the use of injured tissue.

Modulation of Established Inflammatory Pain

It is now established that during inflammation, beyond this initial facilitation on day 1, there is a superseding shift towards descending inhibition of the site of primary hyperalgesia, which acts to limit the impact of accumulating peripheral and central sensitization. First suggested by Schiabe and colleagues, who reversibly spinalized cats using cooling of the spinal cord, it was shown that inflammation induced a progressive enhancement of descending inhibition (Schaible, Neugebauer et al. 1991). This effect was similarly replicated using lidocaine to block descending controls during CFA induced paw inflammation (Ren and Dubner 1996).

Terayama and colleagues showed that this predominance of inhibition does not occur immediately, rather developing after an initial period of facilitation. After the initial increase in current intensity required for complete inhibition of PW observed at the 3rd hour of CFA inflammation there is a decrease over the next 21 hours, shifting the stimulus response curve to the left, indicating the switch to a net descending inhibition (Terayama, Guan et al. 2000, Terayama, Dubner et al. 2002). This leftward shift is similarly replicated in NMDA and AMPA dose-response curves 24 hrs post inflammation (Guan, Terayama et al. 2002). This is indicative of a switch in the RVM to descending inhibition, originating from plasticity at glutamatergic synapses (Vanegas 2004). This plasticity similarly increases the sensitivity of the RVM to opiates (Zhang and Hammond 2010).

Single unit recordings from the RVM similarly support the idea of a shift towards inhibition from the RVM (Miki, Zhou et al. 2002). Continuous recordings over the 3-6 hours after CFA identified a phenotypic switch of neutral cells to pain modulating ON-like or OFF-like cells, as was not seen in naïve animals. This was confirmed with a population study. There was similarly a reduction in the number of OFF-like cells showing a pause of activity after noxious stimulation after inflammation, which together lead to a suggestion that RVM neurons may switch to favour descending inhibition (Miki, Zhou et al. 2002).

This enhancement of descending inhibition is not limited to the RVM however but involves the noradrenergic system too. Using lesioning, Tsuruoka et al suggests that inflammation activates

inhibitory controls originating from the LC to restrict the development of hyperalgesia during inflammation (Tsuruoka and Willis 1996). However, the effect of lesioning are lost by the 7th day of inflammation, whereby no difference is observed between the hyperalgesia between the sham and lesion groups (Tsuruoka and Willis 1996). This suggests that the LC and NA are only involved in descending inhibition during a short initial window of inflammation, as is observed in both the MIA model of OA and tibial nerve injury neuropathic pain model (Hughes, Hickey et al. 2013).

In considering the plasticity of these systems other experiments have shown that, much like in the dorsal horn, there are significant alterations in gene expression and receptor populations over the course of inflammation. Miki and colleagues, in proposing the concept of brainstem sensitization, identified peripheral inflammation induced changes in NMDA receptor gene expression in the RVM (Miki, Zhou et al. 2002). They identified a significant increase in NMDA subunit mRNA over the proceeding 1-7 days after CFA injection, with the greatest increase in NR2A unit mRNA and protein. Similarly, inflammation induces a significant increase in AMPA receptor subunit mRNA, with significant up-regulation of GluR1-flip protein over 24hr-3 days after CFA (Guan, Guo et al. 2003). It is well established that Glutamate plays a prominent part in excitatory transmission in the RVM and activation of descending control from brainstem sites (Aimone and Gebhart 1986, Beitz 1990, Spinella, Cooper et al. 1996), where we have discussed above the shift in the dose response curves for this transmitter during inflammation (Guan, Terayama et al. 2002). As such, these results would suggest that part of the increase in descending control observed during inflammation may originate from an increase in NMDA and AMPA receptor populations, composed of subunits with high conductance properties (NR2A) and a reduced rate of desensitization (GluR1-flip), which increase excitability and activation of RVM neurons. Since the activation of AMPA receptors in the RVM mediate descending inhibition (Urban, Coutinho et al. 1999), the growth of this receptor population in the RVM during the first week of inflammation goes some way to explain the increased descending inhibition observed to be limiting acute inflammatory pain. However, the leftward shift in the dose response curve of AMPA and NMDA in descending inhibition was significant as early as 5hrs after the induction of inflammation when these protein changes only reach significance after 24hrs (Guan, Guo et al. 2003), pointing to additional, faster acting mechanisms of plasticity in the brain stem.

The complexity of the overall pain profile during inflammation is added to by evidence suggesting that primary and secondary sites of hyperalgesia may be differentially controlled (Vanegas 2004). It is suggested that there is an inhibitory drive to the area of primary hyperalgesia, but a facilitatory drive in the surrounding spinal segments that underlie

secondary hyperalgesia and referred pain. This is demonstrated by the ability of lidocaine, NMDA receptor or neurotensin receptor antagonists in the RVM to attenuate the development of secondary thermal hyperalgesia during paw inflammation(Ren and Dubner 1996, Urban, Coutinho et al. 1999, Wei, Dubner et al. 1999). On the basis of this evidence we would expect a descending facilitation of noxious transmission from areas of secondary hyperalgesia, for example the rat paw in a model of knee OA, and inhibitory controls presiding over the joint itself.

When we consider OA specifically, much is yet to be understood about the role of supraspinal controls in OA pain. While some work has been done to characterize how these influences may change during OA, and the consequences this may have on OA pain, the picture is by no means complete. Early work using either cold block spinalization or transection to elucidate the role of descending controls in inflammatory joint pain revealed the increase in descending inhibition which followed in the initial 24hrs(Schaible, Neugebauer et al. 1991, Danziger, Weil-Fugazza et al. 1999). More recently, work from this lab has characterized the chronic shifts in descending controls during the MIA model of OA. They showed adaptive changes in serotonergic controls that may underlie increased evoked responses to dynamic brush and innocuous punctate stimuli(Rahman, Bauer et al. 2009), along side work revealing a time sensitive effects of atipamezole or milnacipran plus atipamezole on evoked responses in the early stages of MIA induced OA(Burnham and Dickenson 2013).

5.1.3 Our Understanding of Brainstem Sensitization in the OA Clinic

The large part of our understanding of brainstem sensitization in the clinic originates from brain imaging processes, notably fMRI. In some of the initial studies characterizing brainstem activation during central sensitization, Zambreau and colleagues demonstrated that following the heat/capsaicin sensitisation model, to induce secondary hyperalgesia, significantly greater brainstem activation was observed in capsaicin sensitized subjects versus controls, specifically in the nucleus cuneiformis (NCF) and PAG, areas with substantial modulatory input into the RVM(Zambreau, Wise et al. 2005). In another study, cardiac-gated fMRI techniques demonstrated changes and differences in brainstem activity during primary and secondary dynamic mechanical allodynia in the capsaicin model(Mainero, Zhang et al. 2007). During dynamic brush stimulation of either the primary or secondary area of mechanical allodynia, increased activity was observed versus controls in the LC and PB. These enhancements in PB and LC processing are understood to represent increased processing of ascending and

descending signals respectively. Similarly, significantly enhanced activity of dorsal reticular nuclei and RVM were observed during stimulation of the primary hyperalgesia sites, though not during stimulation of the secondary areas. This work suggested that brainstem involvement in pain and CS may differ between primary and secondary sites of allodynia, as previously reported in animal work.

Later brain imaging studies have similarly suggested greater activation of the PAG, this time in OA patients receiving punctate stimulation to areas of referred pain vs. controls (Gwilym, Keltner et al. 2009). These works were used to suggest that these spinally projecting brain stem centers might be highly relevant to the generation of the overall OA pain profile, at least playing some important role within the overall CS profile. The study similarly showed a strong correlation between the extent of PAG activation and the degree of CS, as measured by PainDETECT. Such measures support the conclusions of animal work for a crucial role of the brainstem in the overall pain profile.

However, discussions of the inferences of brain imaging work also, rightly, point to the importance of the entire pain matrix feeding into and modulating the brain stem. For example, in a study of visceral pain in IBS patients it was suggested that hypersensitivity related to deficits in inhibitory systems as a result of failed anticipatory down-regulation (Berman, Naliboff et al. 2008) as seen by the lost anticipatory deactivation of areas such as the amygdala or supragenual anterior cingulate cortex in IBS patients when anticipating a painful distention. In essence, it is possible that enhanced pain, as a result of adapted descending control, could be the complex outcome of negative emotional outputs of the pain matrix, not least catastrophising, hypervigilance and anxiety, the consequences of which feed into areas like the RVM (Bingel and Tracey 2008).

It is clear from both pre-clinical and clinical analysis that adaptations, both short and long term, occur across the brain during pain conditions. Previous work in this lab and others has suggested adaptations in RVM cell characteristic firings could be expected during chronic pain. This work sets out to identify whether in this milder model of OA pain adaptations in the magnitude of responses of RVM ON-cells are observed following ipsilateral and contralateral mechanical stimulation; adaptations which could be interpreted as a brainstem manifestation of CS.

5.1.4 Chapter Aims

For patients with symptomatic OA the unmet needs are still clear – efficacious, safe and tolerable analgesia. In part, this is a consequence of the remaining limitations to our understanding of the mechanisms underlying pain in OA.

This study aims to characterize potential adaptations in the response properties of the pain responsive cells in the RVM in a 1mg MIA, non-neuropathic model of OA using electrophysiology. It is hoped this may further our understanding of the supra spinal controls involved in this model of OA pain, and consequently inform our understanding of viable targets for pharmacological intervention. Many previous studies have used shorter-term models so here the impact of OA was examined in the later stages.

5.2 Methods

5.2.1 Animals

All work was conducted in Male Sprague Dawley rats, bred and housed in the Central Biological Services Unit at University College London. As described in Chapter 2, behaviour and electrophysiology was conducted from day 14 onwards in animals weighing 250-300g.

5.2.2 Induction of the model

As detailed in Section 2.2, rats were injected with 1mg MIA (Sigma, UK) to the left knee.

5.2.3 Behavioural Assessment

As described in Section 2.3.3, paw withdrawal was assessed at day 14 using the “up-down method”, as described by Chaplan et al (Chaplan, Bach et al. 1994). In brief, vF hairs of sequential increasing or decreasing force are applied, based on the response to the previous stimuli (withdrawal or lack thereof). The statistical formula described by Dixon et al is then utilized to calculate the 50% withdrawal threshold (Dixon 1980) – which describes the force at which the animal will withdraw 50% of the time. Significant differences in paw withdrawal thresholds analysed using an un-paired students’ t-test.

5.2.4 Electrophysiological Assessment

in vivo electrophysiology was carried out in rats weighing 250-300g at day 14-16 after injection of either 1mg MIA or Saline, as described in detail in Section 2.5.

Once a cell had been identified and classified as either ON-, OFF- or neutral, based on changes in firing prior to tail flick, the cell was characterized through three phases of testing, all described in detail Section 2.5.2:

1. Change in rate of firing in response to tail flick.
2. Mean resting activity over 15 minutes.

3. Change in rate of firing in response to ipsilateral and contralateral stimulation of the paw and knee.

5.2.5 Data Analysis

While recording, individual cells were isolated visually on the oscilloscope and audibly through the speakers, however data was extracted and analysed based upon waveform shape (See 2.5.4). This allowed several cells to be analysed in tandem, for example both the visually identified ON-cell and a neighbouring neutral cell.

The effect of each stimulus, the maximum change in rate of firing, was calculated as:

$$\text{Rate of Firing During/After stimulus} - \text{Mean Baseline Rate}$$

Where baseline was calculated as the mean rate of firing for the 20 seconds directly preceding the stimulus; Rate of firing during was calculated as the mean rate of firing for the 20seconds during von Frey or clamp application; and the rate of firing after stimulus was the mean rate in the 20seconds after the stimulus. The greatest change in rate of firing over the three rounds was used for analysis. This measure of after stimulus change in rate of firing was calculated following observations that the increase or pause in firing sometimes followed the *release/removal* of the more noxious stimuli, namely the knee clamp and 60 and 100g von Frey hairs.

Change in rate of firing was analysed using column statistics, namely to calculate the 25 percentiles, mean and standard deviation, alongside a One-sample t-test to determine if the mean change in rate of firing was significantly different from 0, denoting no change, and a Wilcoxon Signed rank test to determine if the median change in rate of firing was significantly different from 0.

As described in Sikandar et al(Sikandar, Bannister et al. 2012), ON-cells were then formally reclassified by their response to paw and knee stimulation. Briefly, the mean percentage change in firing in response to a stimulus was calculated using the formula:

$$((\text{Mean rate of firing after the stimulus} / \text{mean baseline rate of firing}) * 100) - 100$$

where percentage changes >15% identified ON-like responses; > negative 15% identified an OFF-like response; and responses between 15% to -15% identified neutral-like responses.

In all graphs, data is expressed as the change in rate of firing, spikes per second, plotted as either scatter plot; box and whisker; or as the mean change in rate of firing (spikes per second) \pm SEM. Significant differences in the extent of change in rate of firing between groups and sides (i.e. MIA vs. Sham animal; Ispi vs. contra) was determined using a non-parametric Mann-Whitney U test.

5.3 Results

5.3.1 Recording Sites

Bearing in mind the limitations of assuming that the recording site coordinates truthfully correspond to the rat atlas defined location without histological confirmation, it appears likely that a large number of cells fall within the nucleus reticularis gigantocellularis, commonly abbreviated to either RGC, NGC, or Gi – as it is referred to in this copy of the rat atlas – and not the desired RVM nuclei of the NRM, nucleus reticularis paragigantocellularis, or nucleus reticularis gigantocellularis pars alpha (NGC α).

Analysis within this chapter, and a large portion of the discussion, proceeds from the perspective of the expected cell types found in the RVM, on the understanding that ON cells as defined in the RVM have also previously been characterized in the NGC (Fields, Bry et al. 1983). The limitations and contrary implications of potentially having recorded from the NGC will also be considered throughout, and in greater depth in sections 5.4.1 and 5.4.5.

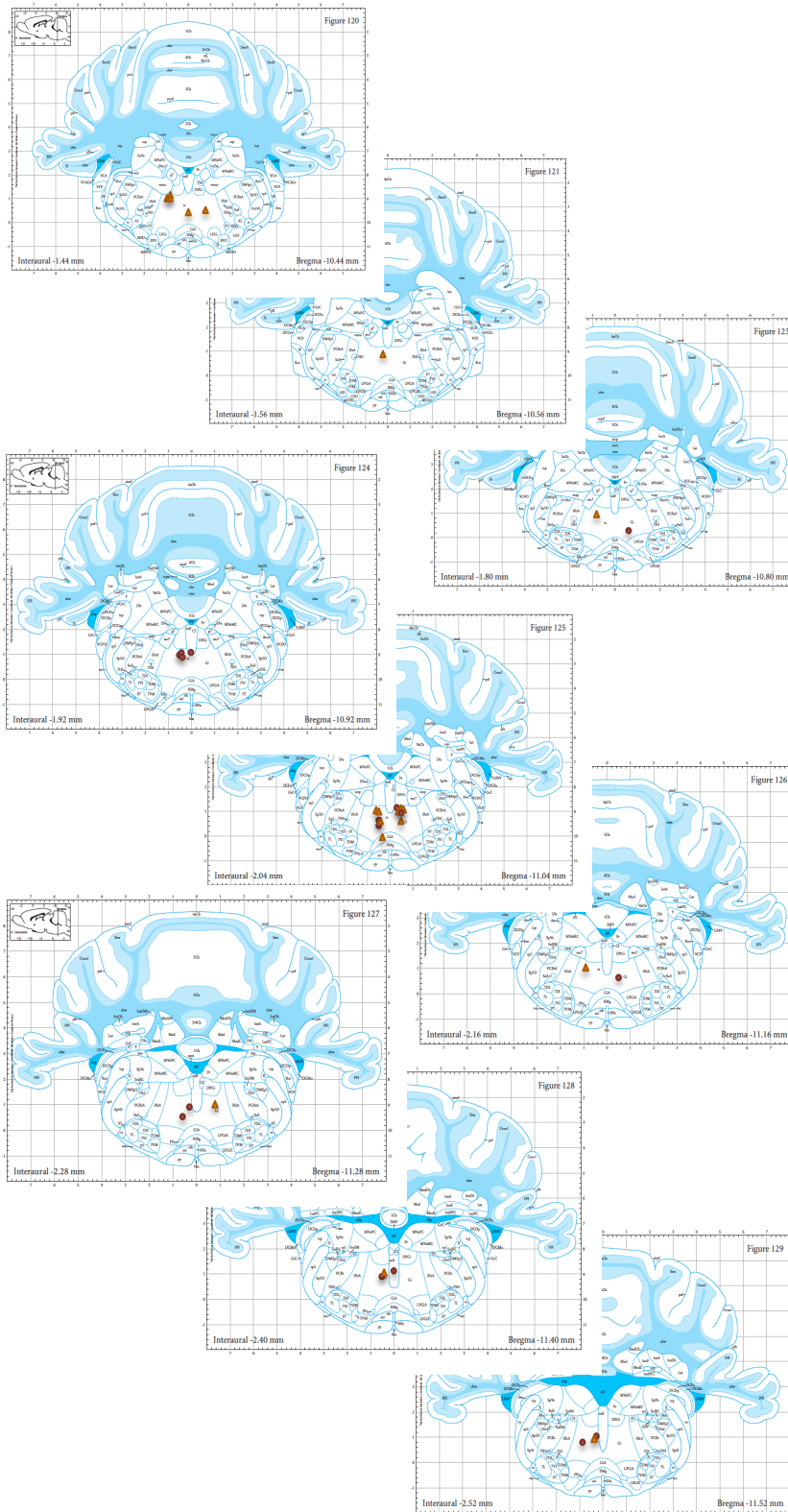


Figure 5.2 - A diagrammatic representation of coronal sections corresponding to RVM nuclei with recording sites indicated. Coronal depth is evaluated by the dorsoventral distance from the horizontal plane passing through bregma and lambda on the surface of the skull. **Red circles** indicate recording sites in MIA rats. **Orange triangles** indicate recording sites in sham rats

5.3.2 Confirmation of behavioural hypersensitivity

The 50% mechanical paw withdrawal threshold (PWT) of the ipsilateral and contralateral paw was assessed in rats 14 days after the intra-articular injection of 1mg MIA and compared to the

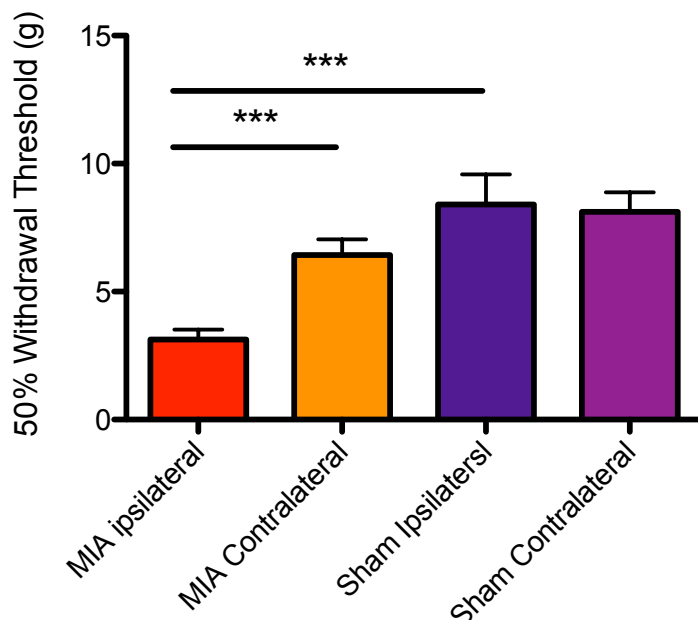


Figure 5.3 – Monosodium Iodoacetate induced OA produced significant mechanical hypersensitivity in the ipsilateral paw versus both contralateral paws and sham animal controls. At Day 14 following injection, MIA animals exhibit a significant reduction in the 50% withdrawal threshold to vF hairs applied to the paw, versus both contralateral paw and sham ipsilateral paw WT (MIA n=13, Sham n=15; *** p≤0.001)

sham control, which received saline. The significant reduction in the 50% PWT on the ipsilateral side in MIA animals, versus both contralateral paw and sham control animals, indicate the development of a marked ipsilateral mechanical hypersensitivity (Figure 5.3; n=15-16; Unpaired Students T-test; *** p≤0.001).

While no significant difference is observed, there is similarly an interesting trend towards a reduction in the MIA contralateral paw

WT compared to the sham ipsilateral and contralateral PWT's, for example MIA CL: 6.4 ± 0.6 g versus Sham CL: 8.1 ± 0.8 g. Similarly, no difference in PWT was observed between Sham ipsilateral and contralateral paws.

Table 5.1 – Total animals used and cells characterized within each condition

Condition	Number of Animals	ON Cells	OFF Cells	Neutral Cells
MIA	13	20	1	12
Sham	15	23	6	9

5.3.3 Electrophysiological Recordings in MIA and Sham Rats

A total of 13 MIA and 15 Sham animals were used, from which 20 and 24 ON cells, 1 and 6 OFF cells and 12 and 9 neutral cells were characterised respectively (Table 5.1). The number of OFF cells is in contrast extremely low, notably in the MIA group, following continuing difficulties in

identifying OFF cells in the MIA animals, despite perseverance, and so the decision was made to discontinue the search, in line with our 3Rs obligations. However, given the volume of ON cells characterised it was felt this would be sufficient to identify any alterations in firing patterns in these animals, and thus ON cells are the focus on the analysis in this study.

Example electrophysiological recordings in Figure 5.4 illustrate selected ON, OFF, and neutral cells showing characteristic changes in reflex-related firing following the application of the noxious heat stimulus to tail. Those identified as ON-cells increased reflex-related firing (Figure 5.4A); OFF-cells paused reflex-related firing (Figure 5.4B); and neutral cells show no observable meaningful change in reflex-related activity (Figure 5.4C).

Following the initial electrophysiological identification of the ON, OFF or Neutral cell type, the baseline rate of firing, change in rate of reflex related firing and time delay (latency) to tail flick following immersion in hot water were characterized (Figure 5.5). No significant difference was observed between the baseline rate of firing of either ON or Neutral cells in MIA vs. Sham (OFF could not be assessed) (Figure 5.5 A). However, there is a visible trend towards decreased baseline firing rates in both MIA ON and MIA neutral cells in comparison to Sham ON and neutral cells: for example ON cell rate of firing 2.8 ± 1.0 spikes per second vs. 7.2 ± 2.2 spikes per second in MIA vs. Sham. Similarly, no significant difference was observed in the latency to tail flick following the 8cm immersion of the tail in 50°C water (Figure 5.5B), nor significant difference in the mean change in rate of firing in ON or neutral cells in relation to the reflex tail flick (Figure 5.5 C) between sham and MIA animals.

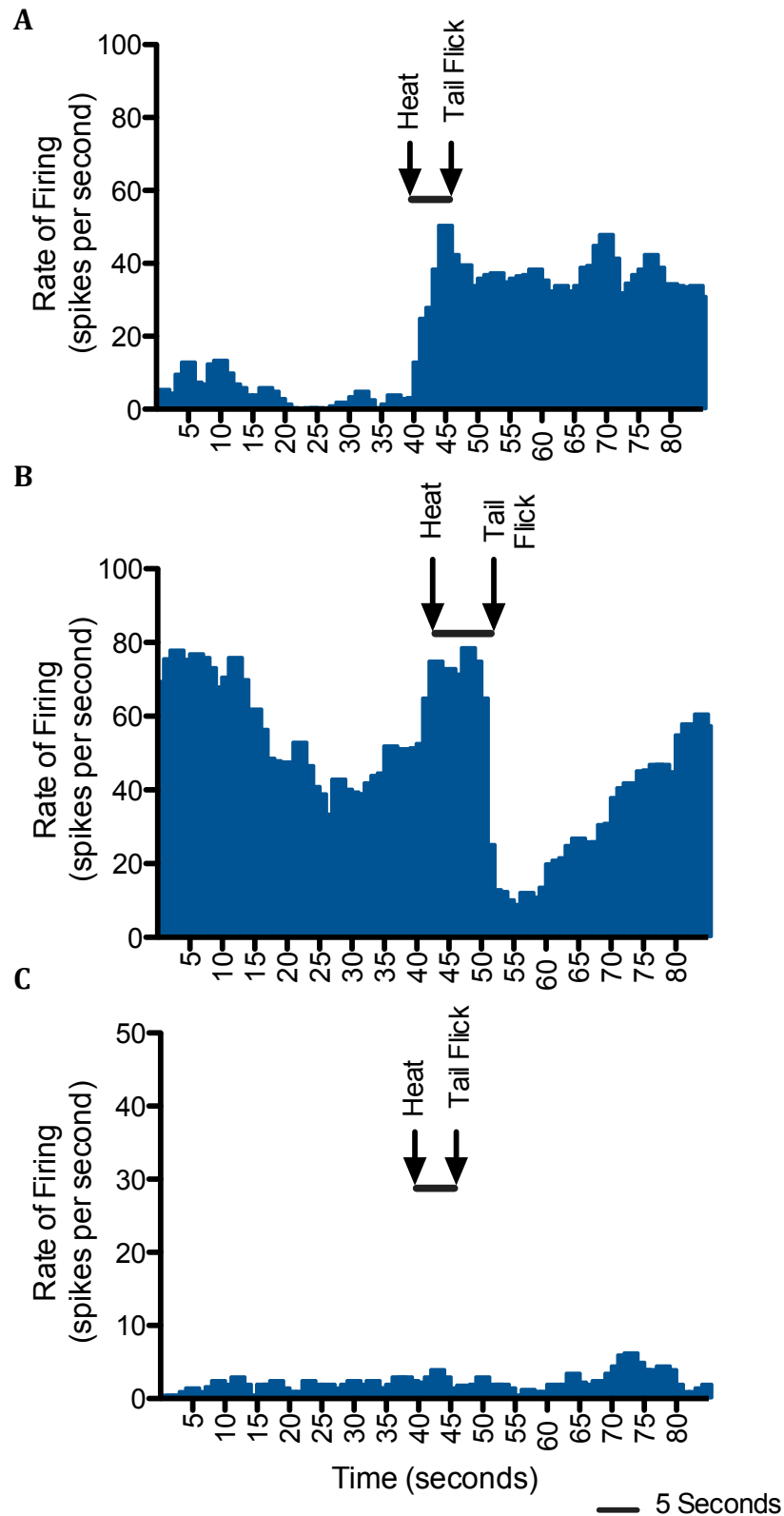


Figure 5.4 - ON, OFF, and neutral cells display characteristic changes in reflex-related firing following a noxious somatic stimulus. A) Example trace of an RVM ON-cell that increases reflex-related firing following noxious tail heat (↓Heat = application of heat stimulus, ↓Tail flick = visible initiation of tail flick). **B)** Example trace of an RVM OFF-cell that pauses reflex-related firing (spikes) following noxious tail heat. **C)** Example trace of a NEUTRAL-cell that displays no consistent change in reflex-related activity following noxious tail heat.

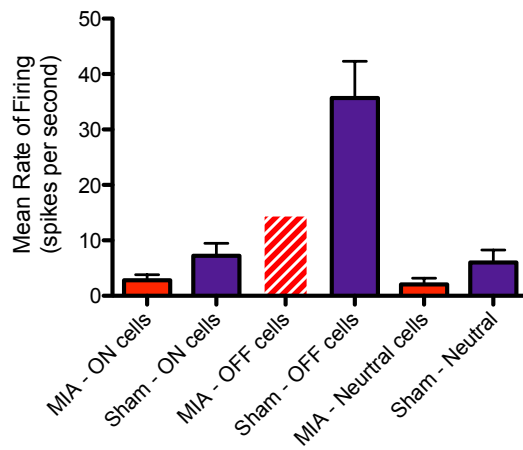
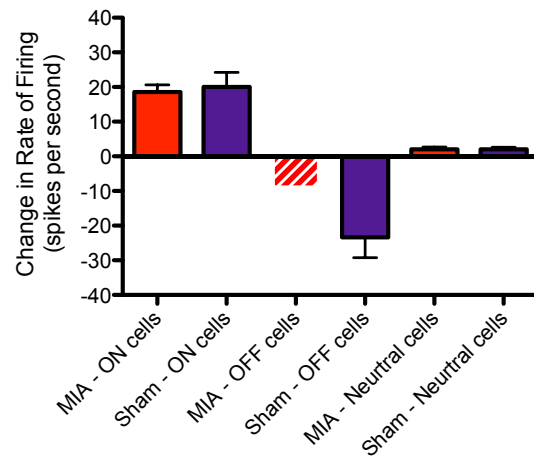
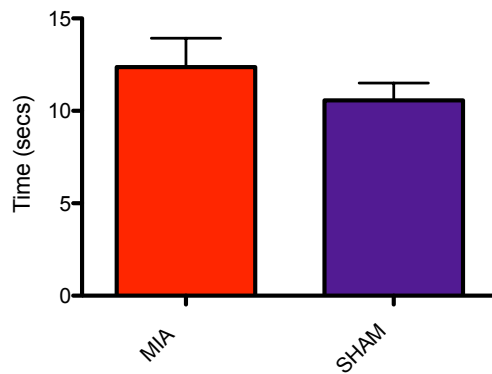
A**B****C**

Figure 5.5 - The baseline rate of firing, latency to tail flick following application of noxious tail heat, and change in rate of reflex related firing of ON, OFF and Neutral cells did not significantly differ between MIA and Sham animals 14-16days following injection. A) No significant difference was observed between the baseline rate of firing of ON, OFF and neutral cells (Mean over a 15minute period) in MIA versus sham animals. **B)** No significant difference was observed in the mean change in rate of firing, out of three responses, upon tail flick reflex response in ON, OFF and neutral cells in MIA and Sham animals. **C)** No significant difference was observed between MIA and sham animals' latency to tail flick upon the 8cm immersion in 50°C water.

5.3.4 Characterization of ON cell responses to somatic stimulation

The change in rate of firing of previously identified ON cells was then characterized following the application of noxious and non-noxious stimuli to the IL and CL paw and knee in MIA and Sham animals. The change in rate in the 20s following the release of the stimuli was used for further analysis as both through experimental observation and data (See Appendix) this often produced the greatest change in nociception-related firing.

In contrast to the responses to noxious tail heat, these mechanical somatic stimuli did not produce consistent changes in activity following noxious and non-noxious mechanical stimulation of the paw and knee, in both MIA and sham animals. As can be seen in Figure 5.6 and 5.7, while the large proportion of cells increased their firing, a small number exhibited reduction in firing, clearly observed in response to ipsilateral knee pinch in MIA animals (Figure 5.6A and 5.7A).

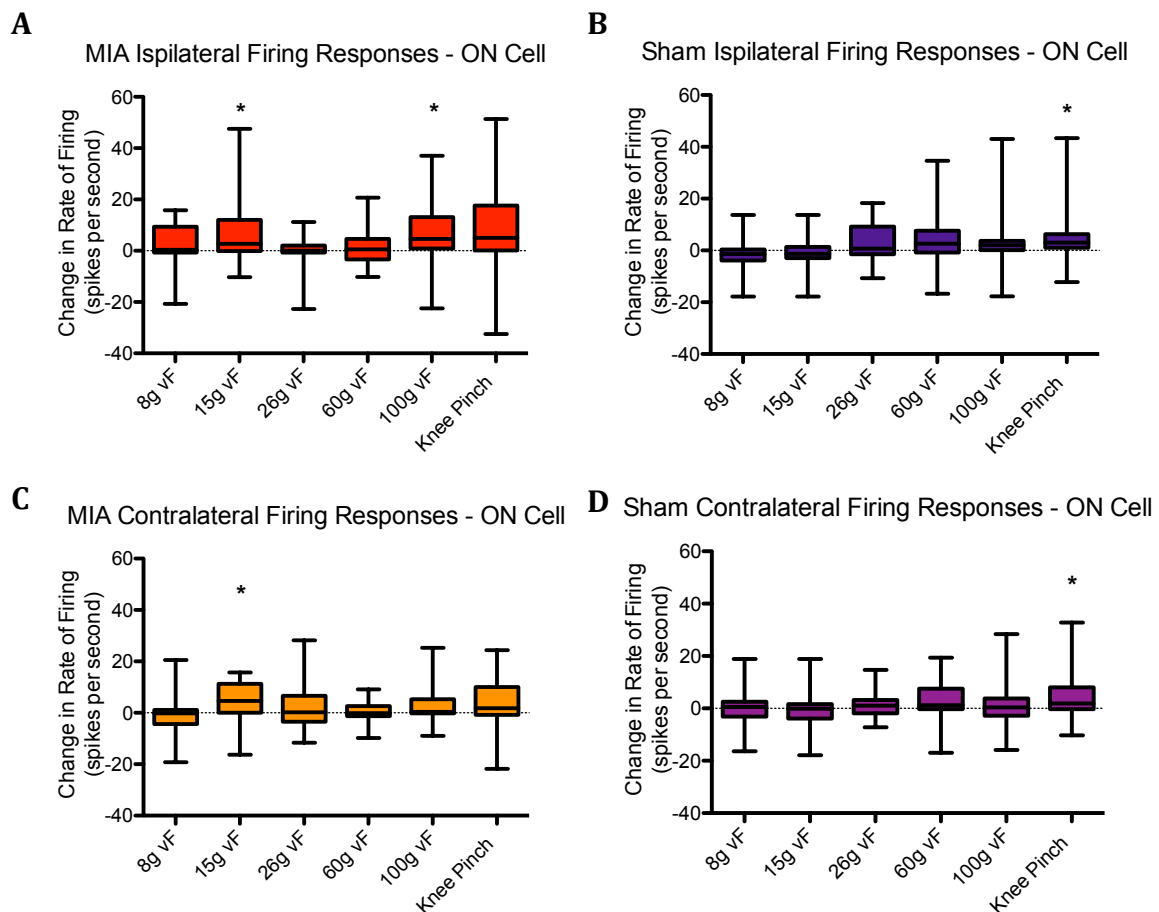


Figure 5.6 - The response of ON-cells to mechanical stimulation to the knee and paw following MIA and saline produced divergent responses, with a minority of cells responding in an OFF like manner to reduce the rate of firing. A+C) Response of ON-cells in MIA animals to ipsilateral (A) and contralateral (C) knee pinch and vF application to the paw, in which only the responses to 100g in IL and 15g vF in IL and CL were significant different from 0 (significant change in rate). B+D) Change in rate of firing in ON-cells in Sham animals to ipsilateral (B) and contralateral (D) stimulation, where only the knee pinch evoked a significant change in firing. (* $p \leq 0.05$).

Across all groups, column statistics were performed, box-and-whisker diagrams generated and the significance of change in rate of firing versus 0, or no change, calculated (Figure 5.6; One sample T-test; * $p \leq 0.05$). In MIA animals, no significant change in rate of firing was observed across most stimulus groups, with the exception of 15g vF in both IL and CL paw and to 100g vF in IL paw. The stimulation of the IL side trended towards a greater increase in firing, with a firing pattern similar to a stimulus intensity coding in the MIA IL group (Figure 5.6A) – where the less noxious and non-noxious stimuli responses cluster closer to zero, with the exception of 15g vF, such that more noxious stimuli evoke greater changes in the rate of firing.

In Sham animals, a significant change in the rate of firing is only observed in response to knee pinch in both IL and CL stimulation, though there is a trend towards an increased rate of firing in response to both 60g and 26g vF (60g: IL $p=0.11$, CL $p=0.09$; 26g IL $p=0.07$; One sample T-test).

Overall the change in rate of firing is smaller in the sham animals than MIA animals, and on the contralateral side versus the ipsilateral side. Change in firing to the non-noxious and less noxious stimuli clustered near 0 – indicating a lack in response to stimulation (Figure 5.6 and 5.7).

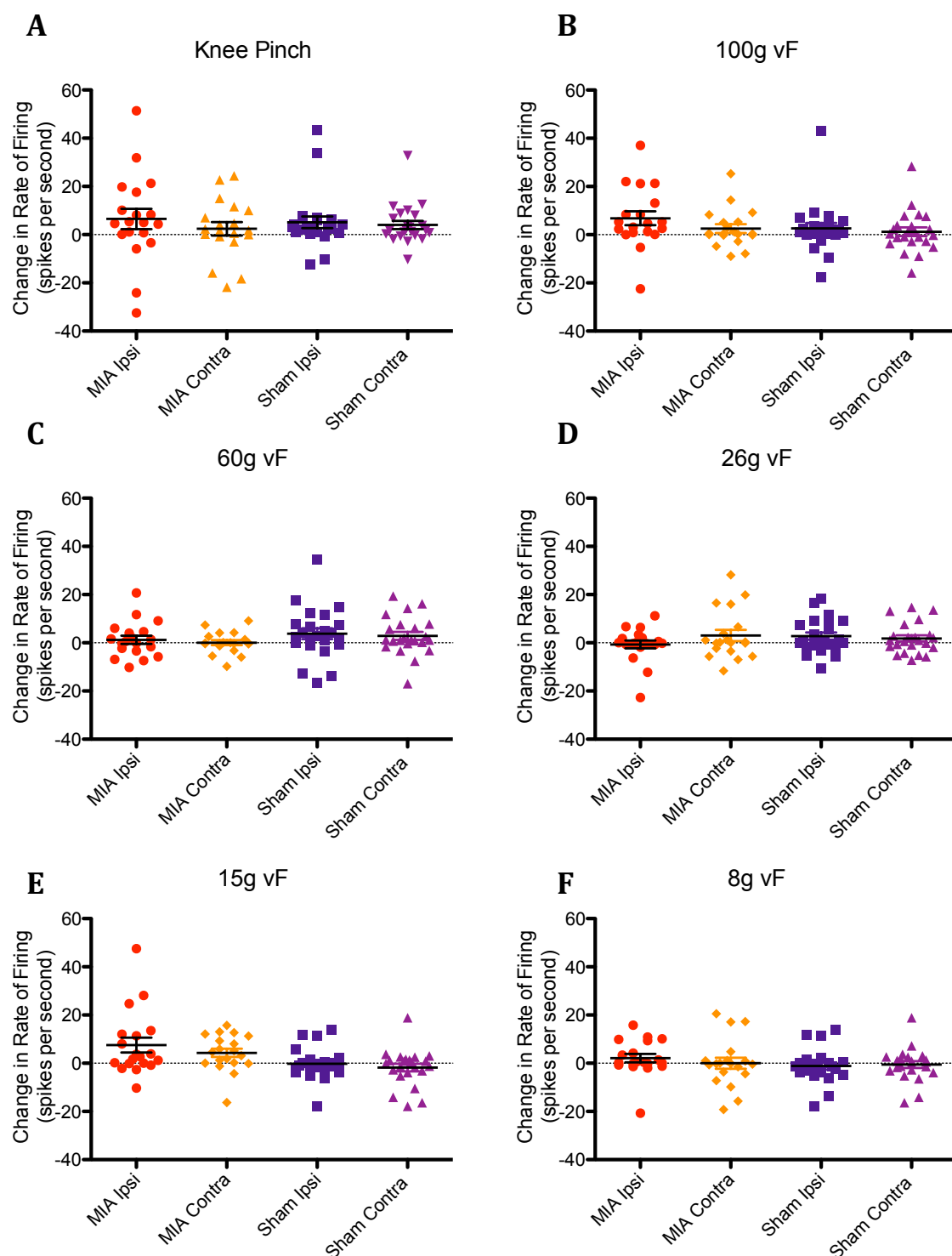


Figure 5.7 – The response of ON-cells to mechanical stimulation to the knee and paw 14-16days following injection of either 1mg MIA or saline produced divergent responses within groups, with a minority of cells responding in an OFF-like manner to reduce the rate of firing. A) Knee Pinch B) 100g vF C) 60g vF D) 26g vF E) 15g vF F) 8g vF

5.3.5 Reclassification and ON:ON-like cell analysis

As can be observed in Figure 5.6 and 5.7, a proportion of ON cells (classified earlier by their response to noxious heat induced tail flick) altered their firing in a manner inconsistent with this classification.

Table 5.2 – Changes in activity of ON-cells following the application of 8-100g vF hairs or knee pinch (KP) to the ipsilateral or contralateral paw 14-16days after the injection of MIA or Saline: Responses were either classified as ON-like (>15% increase in firing), OFF-like (>15% decrease in firing) or Neutral-like (<15% change in either direction) based on the change in rate of firing following stimulus compared to baseline

	MIA IPSILATERAL						MIA CONTRALATERAL					
	8	15	26	60	100	KP	8	15	26	60	100	KP
ON-LIKE	7	12	7	8	16	14	8	13	9	7	8	11
OFF-LIKE	4	2	9	9	0	3	9	5	9	7	5	4
NEUTRAL	8	5	3	2	3	2	2	1	1	5	6	4
	SHAM IPSILATERAL						SHAM CONTRALATERAL					
	8	15	26	60	100	KP	8	15	26	60	100	KP
ON-LIKE	12	6	12	13	14	18	9	7	8	14	9	14
OFF-LIKE	5	9	7	5	2	3	5	7	9	6	8	6
NEUTRAL	6	8	4	5	7	2	9	9	6	3	6	3

Using analysis previously described by Sikandar et al (Sikandar, Bannister et al. 2012), these cells were reclassified based on the </>15% change in the rate of firing following the application of noxious and non-noxious stimuli to the knee and paw, such that cells would be described as ON-like, OFF-like or neutral.

As described in Table 5.2, this resulted in a proportion of ON cells being classified as OFF-like or neutral-like in their responses to stimulation of the paw or knee, though the majority were ON-like responses. The total number of ON-like responses is consistent across groups, such that 56%, 49%, 54% and 44% of responses are classified as ON-like in MIA IL, MIA CL, Sham IL and Sham CL respectively. This proportion is marginally higher in response to ipsilateral stimulation, where fewer OFF-like responses are similarly observed.

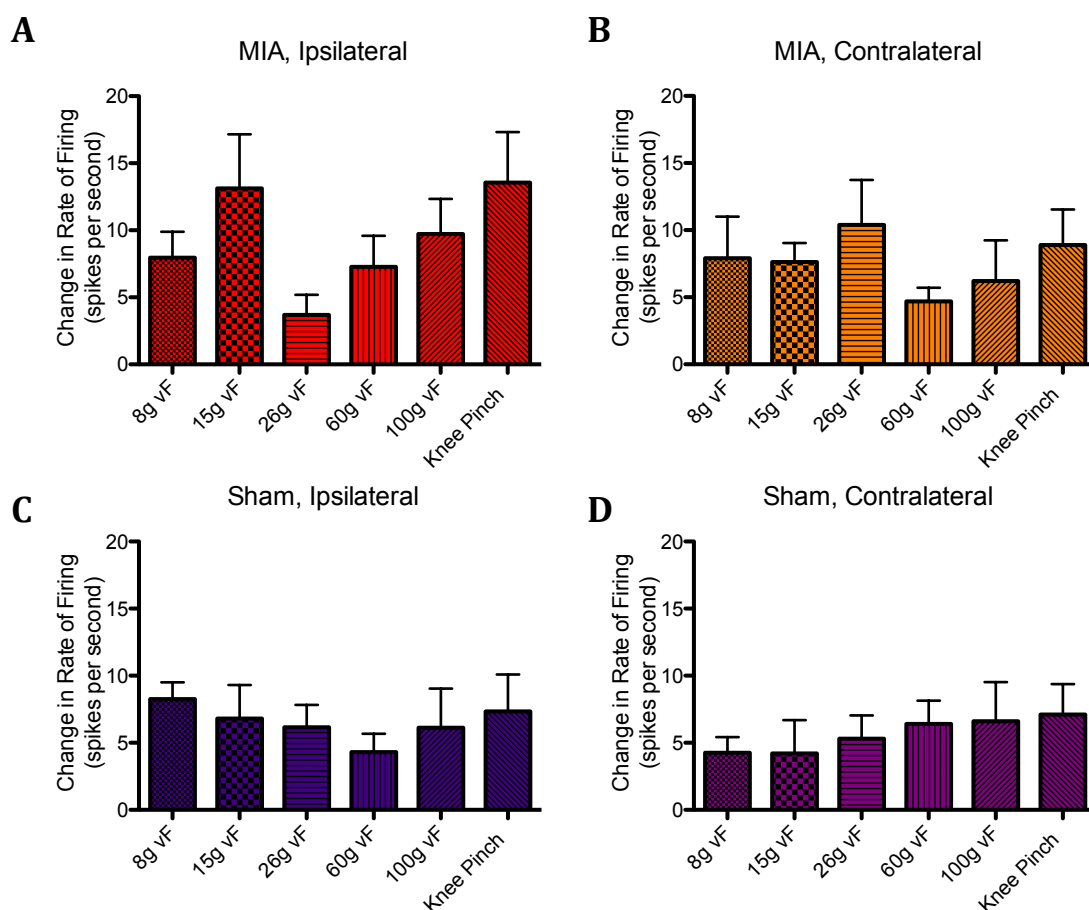


Figure 5.8 – Change in the rate of firing of ON-Cells exhibiting ON-like changes in the rate of firing following the application of von Frey hairs or knee pinch to the ipsilateral or contralateral hind limb 14-16days after the injection of 1mg MIA or saline (Sham). A+B) ON-like changes in rate of firing in RVM ON-cells in MIA animals following mechanical stimulation of ipsilateral (A) and contralateral (B) paw and knee; and in sham animals following ipsilateral (C) and contralateral (D) stimulation

ON cell responses were most consistent in response to the most noxious stimuli, such that a greater proportion of cell responses were classified as ON-like in response to 60g, 100g vF and knee pinch, with relatively few OFF-like responses observed to these most noxious stimuli in ipsilateral knee and paw (both MIA and Sham). Overall, the greatest number of neutral-like responses were observed in response to the non-noxious and mildly noxious stimuli of 8g and 15g vF – with the exception of MIA CL – however, this is to be expected since ON-like responses would only be expected to stimuli sufficient to evoke a reflex withdrawal, where a lack of response results in a Neutral-like classification.

The magnitude of change in rate of firing in ON cells responding in an ON-like manner to mechanical stimulation of the paw and knee are outlined in Figure 5.8 and 5.9. No significant differences are observed between either group (MIA vs. Sham), side (IL vs. CL) or stimulation force (8-100g vF hairs) respectively across these figures, however trends are apparent.

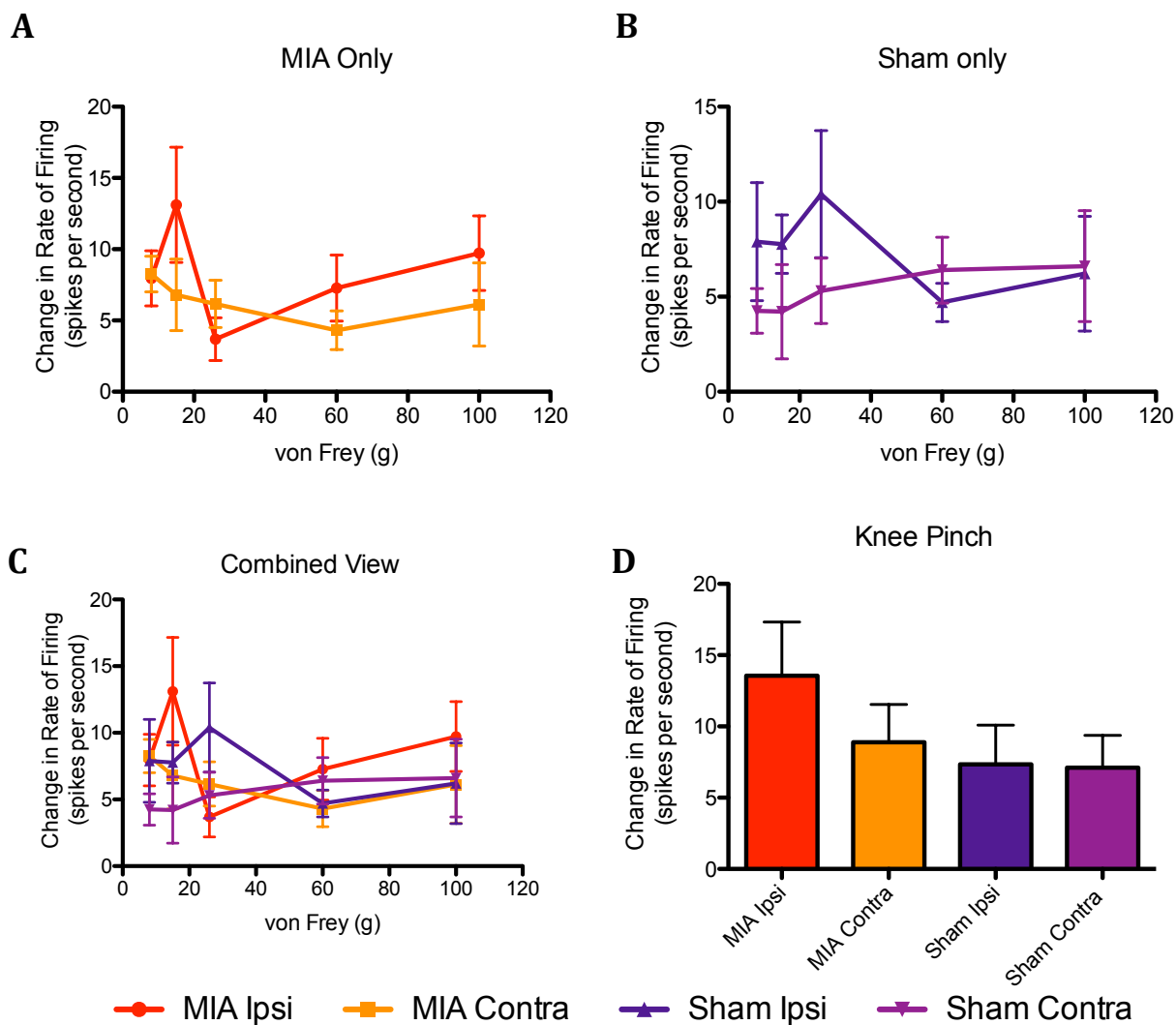


Figure 5.9 - The ON-like response of recorded ON-cells to mechanical stimulation to the knee and paw 14-16 days after injection of either 1mg MIA or saline. Evoked responses of MIA (A) and sham (B) treated animals to ipsilateral and contralateral von Frey to the paw, and in merged view (C). D) Evoked response to knee pinch

Consider Figures 5.8A and Figure 5.9A and C. Though not significant, there is an apparent trend towards a greater increase in the rate of firing following ipsilateral MIA knee pinch, 60g, 100g and 15g vF hair application to the paw versus contralateral and sham stimulation.

In contrast with a relatively homogenous extent of change, the response to ipsilateral 15g vF in MIA animals equivalent to that of the knee pinch. While 15g vF wouldn't traditionally be considered as strongly noxious, this is beyond the 50% withdrawal threshold observed in these animals (Figure 5.3) and thus sufficiently noxious to MIA animals to evoke a strong withdrawal, the behavioural correlate of changes in ON cell firing.

5.4 Discussion

Changes to the rate of firing of ON and OFF cells in the RVM are by now well characterized, such that we expect distinctive pauses in firing from OFF cells and a rapid enhancement in the rate of firing from ON cells after the application of a nociceptive stimuli sufficient to evoke a reflex withdrawal (Fields, Bry et al. 1983, Heinricher, Barbaro et al. 1989). It is similarly well characterized through spinal electrophysiology and pharmacology that monoamine controls descending from the brainstem, including the RVM, act to modulate nociceptive transmission in the spinal cord (Stanfa and Dickenson 1994, Green, Lyons et al. 1998, Green, Scarth et al. 2000, Suzuki, Rygh et al. 2004, Rahman, Bauer et al. 2009, Sikandar, Bannister et al. 2012, Burnham and Dickenson 2013, Bannister, Patel et al. 2015). The plasticity in brainstem processing during chronic pain conditions, and the roles in determining the severity, profile, time course and spatial mapping of the pain profile, are increasingly understood in neuropathic nerve injuries (Burgess, Gardell et al. 2002, Vanegas and Schaible 2004, Chen, Oatway et al. 2009, De Felice, Sanoja et al. 2011, Hughes, Hickey et al. 2013, Wang, King et al. 2013). However, less well understood is what these changes, if changes are apparent at all, might look like in an OA animal.

Clinical studies have put forward evidence that central sensitisation spreads beyond the spinal cord to encompass the brainstem during OA in patients (Gwilym, Keltner et al. 2009). While the work presented herein fails to classify significant changes in nociceptive processing in the brainstem of 1mg MIA animals, apparent trends within the data may suggest more subtle adaptations in this model of OA pain.

5.4.1 Implications of recording from NGC

The RVM in the rat is composed of the nucleus raphe magnus (NRM) and adjacent reticular areas, namely the nucleus reticularis gigantocellularis pars alpha (NGC α) and the nucleus paragigantocellularis lateralis (Fields, Bry et al. 1983, Bouhassira, Bing et al. 1993). Directly adjacent to the NGC α sits the NGC. As alluded to in section 5.3.1, it is clear from the recording plots in Figure 5.2 that a majority of cells recorded in this study likely fell within this latter nucleus, thus recording outside of the RVM.

The NGC is sometimes misquoted as forming part of the RVM (Zhuo and Gebhart 1992, Zhuo and Gebhart 1997), in part due the number of similarities between the NGC and nuclei of the RVM. For example, NGC neurones respond to noxious stimulation with increasing, decreasing, or

no change to the rate of firing(Pearl and Anderson 1978, Harris and Sinclair 1981, Mohrland, McManus et al. 1982, Morrow and Casey 1983). Indeed, Fields et al 1983 recorded from what he referred to as ON-cells within the NGC, though no OFF cells were identified during the study(Fields, Bry et al. 1983). Studies have similarly documented the effects of ES, pharmacological and lesioning interventions that highlight a role for the NGC in descending facilitation of spinal processing(Zhuo and Gebhart 1991, Zhuo and Gebhart 1997, Wei, Dubner et al. 1999, Terayama, Dubner et al. 2002, Da Silva, DeSantana et al. 2010), though studies disagree over the exact role of NGC. These include theories of direct, to lamina VII, VIII and IX,(Matsuyama, Ohta et al. 1988) and indirect to lamina I, II and V(Basbaum, Ralston et al. 1986, Jones and Light 1990) via RVM(Beitz 1982, Zagon and Bacon 1991), influences on spinal cord excitability; tonic inhibition of NRM OFF cells via local GABA interneurons(Wei, Dubner et al. 1999); and disinhibition of the A7 noradrenergic descending inhibitory system(Clark and Proudfit 1991, Wei, Dubner et al. 1999). However, in contrast to the RVM the NGC additionally has a role in escape behaviour(Casey 1971), and the incidence of OFF-like cells is much smaller(Pearl and Anderson 1978, Harris and Sinclair 1981, Fields, Bry et al. 1983).

What is the impact to this work of potentially having recorded largely from the NGC? The study remains relevant, given the previously mentioned influence of NGC in pain and spinal excitability. Similarly, the previously established presence of ON cells in the NGC by Fields allows the current analysis to proceed. However expectations for the potential adaptations in neuronal activity may differ – for example, in contrast to RVM it is less clear if the NGC 's cells adapt their baseline firing patterns during pain conditions(Pertovaara and Tukeva 1989, Robinson, Calejesan et al. 2002).

5.4.2 The Baseline Rate of Firing and Latency to Tail Flick in MIA Rats

In line with the trend towards a greater latency to tail flick, the baseline spontaneous activity of the ON-cells identified in these 1mg MIA animal's trends towards decreased rate of firing versus sham animals. Though not significant, at $p=0.119$, this baseline activity is noticeably different and could be taken to indicate a mild resting state of anti-nociception in comparison to sham animals that had not received 1mg MIA to the knee 14-16days prior.

Considered from the perspective of recording in the RVM, the precedent for changes in the baseline rate of firing of ON and OFF cells, and the tangible impact upon reflexes and nociceptive thresholds, is clear. Changes to the spontaneous baseline rate of firing in chronic pain have been observed in animals during both inflammatory and neuropathic pain

models(Khasabov, Brink et al. 2012, Silva, Amorim et al. 2013), though this is not universally observed(Carlson, Maire et al. 2007). It is likely that these changes in the spontaneous rate of firing of ON cells are time sensitive, much like the changes in the balance of descending inhibition and facilitation(Terayama, Guan et al. 2000, Terayama, Dubner et al. 2002, Vanegas and Schaible 2004), since Gonçalves et al 2007 demonstrated enhanced spontaneous activity of RVM ON cells at weeks 1 but not 8 in rats following the SNI model of neuropathic pain(Gonçalves, Almeida et al. 2007). Similarly, the most powerful analgesic agents, opiates, causes significant reduction in the baseline firing of ON cells while enhancing the activity of OFF cells(Heinricher, Morgan et al. 1994). Both the systemic or iontophoretic administration of morphine reduce spontaneous and nocifensive reflex related firing of ON-cells at doses which inhibit noxious heat related tail flick(Barbaro, Heinricher et al. 1986, Heinricher, Morgan et al. 1992). As such, the concept of adaptations in the basal rate of firing in ON cells, or that those reductions may result in tonic anti-nociception and prolong the latency to tail flick are not new ideas. However, three issues are clear here. The first, that observed adaptations in ON cell spontaneous activity are largely enhancements of ON-cell firing in both inflammation and neuropathy(Gonçalves, Almeida et al. 2007, Khasabov, Brink et al. 2012, Silva, Amorim et al. 2013), while these 1mg MIA animals are exhibiting reductions in the basal rate of ON cell activity. Second, these changes are not themselves significant, nor are the behavioural correlates. Thirdly, conclusions are limited severely without the ability to draw conclusions on OFF cell activity. Notably, the first and last of these may in part be addressed by analysis of this data as NGC, not RVM.

On the first: though increases in ON cell firing in the RVM is most commonly observed during pain states, as already described above and discussed in Chapter 4, these changes and the balance between descending facilitation and inhibition have been shown to be time dependent(Gonçalves, Almeida et al. 2007). This trend towards decreased firing in the 1mg MIA model at 14-16days after injection by ON-cells may reflect a time sensitive, disease severity specific point of minor (non- significant) tonic analgesia. This idea aligns well to previous work which shows a time sensitive effect of 5HT₇ antagonism on the reversal of the effects of milnacipran in the 2mg MIA model(Burnham and Dickenson 2013) It may be that significant pharmacological effects are observed by Burnham et al 2013, indicating descending serotonergic inhibitions, due to differences in the severity of the 1mg and 2mg models (See discussion in Chapter 3&4). Conversely, this may simply relate to recording site expectations. While RVM cell adaptations during pain states are well characterized(Gonçalves, Almeida et al. 2007, Khasabov, Brink et al. 2012, Silva, Amorim et al. 2013), it may be that the NGC adapts differently. By recording from NGC, ES studies have previously established that the responses

of NGC cells are inhibited when PAG is stimulated(Harris and Sinclair 1981, Mohrland, McManus et al. 1982, Morrow and Casey 1983) Given the monosynaptic pathway between PAG and NGC(Harris and Sinclair 1981),it is possible that in this 1mg model the PAG has been recruited and is providing some minor tonic inhibition, reflective of a similar spinal effect observed by Burnham in the 2mg MIA model(Burnham 2012).

That no significant difference is observed in the latency to tail flick in MIA and sham animals does not necessarily discredit or rule out the presence of a tonic descending inhibitory influence in these animals. Spinal excitability, a crucial determinant of reflex latency, is a complex outcome of several factors, not least peripheral inputs, a state of central sensitization and a balance between descending facilitation and inhibition. It has previously been suggested that during inflammation, while a minor descending inhibitory drive may exist to neuron pools serving secondary sites, facilitation predominate(Vanegas and Schaible 2004). It is similarly understood that while inhibition from noradrenergic nuclei curb nociception initially, this effect diminishes over time(Burnham and Dickenson 2013, Hughes, Hickey et al. 2013), which could render descending modulation mediated by the RVM and NGC (Zhuo and Gebhart 1990, Zhuo and Gebhart 1991, Zhuo and Gebhart 1992) less physiologically relevant. It may similarly be the case that there is no trend at all, and to look for one is to over interpret no significant work. It may well be that, be this the RVM or the NGC, there is no significant change in the basal activity of these neurones and so no significant behavioural correlate, in the form of latency to tail flick, would be expected.

Thus, the final issue is the strength of any conclusions drawn from this study without the analysis of OFF cell changes between MIA and Sham rats. As outlined in Table 5.1, while a total of 20 and 23 ON cells were identified in MIA and sham rats respectively, only 1 and 6 OFF cells were identified in these animals – despite the use of 28 animals in total. The difficulty in identifying OFF cells may be attributed to the likely recording location, within the NGC. Previous work by Fields et al 1983 concluded that while ON cells are relatively wide spread in the NGC, no OFF cells could be identified (Fields, Bry et al. 1983). In work by Pearl and Anderson et al 1978, in which the response patterns of 162 neurones in the NGC of 31 anaesthetized cats were characterized, only 6% (5 cells) were characterized as suppressing activity following the application of spinal noxious stimuli, in contrast to 76% which demonstrated some form of clear excitatory response(Pearl and Anderson 1978). Harris and Sinclair 1981 concluded similarly in the rat, where single unit recordings in the NGC characterizing the response of cells to noxious radiant heat provided to the tail. The study categorized just 17 of 128 neurones as responding to the radiant heat with a reduction of activity, versus 62 excitatory and 44 none (neutral like) responses(Harris and Sinclair 1981).

As such it seems likely that in NGC the expected ratio of ON:OFF:Neutral like cells favours neurones with a profile similar to ON Cells, as has been characterized in this study.

5.4.3 Physiological Responses Of ON-Cells To Mechanical Stimulation Of The Paw And Knee

Following the classification of cells as ON based upon the change in firing before heat evoked tail-flick, the responses of these cells to escalating force von Frey hairs and noxious knee pinch were shown to be more variable than uniform ON-like responses. While reflex related responses were not expected to all these stimuli, given the innocuous nature of the lower force von Frey hairs, it was expected that tail flick classified ON cells would similarly exhibit stimulus related enhancements in the rate of firing to noxious stimulation like knee pinch or 100g vF. However, as demonstrated in Figure 5.5 and 5.6, this was not the case. Though the majority demonstrated increases in the rate of ON-cell firing, subgroups of cells either did not respond or in fact decreased the rate of firing. Using a classification based on 15% changes in the rate of firing, as previously used in Sikandar et al (Sikandar and Dickenson 2011), these previously identified ON-cells could be reclassified into neutral like and OFF-like cells, as seen in Figure 5.4.

While the absence of change in rate of firing in some neurones and the resultant Neutral-like classification is explained simply, the OFF-like behaviour is less simply explained. This original definition of an ON, OFF or neutral cell relate to the change or lack thereof in the rate of firing just prior to the initiation of nociceptive reflex (such as tail flick or paw withdrawal) (Fields, Bry et al. 1983, Heinricher, Barbaro et al. 1989). As such, changes in firing relate to the application of stimuli noxious enough to provoke a reflex withdrawal, where this threshold will naturally vary between animals and depend on both the extent of sensitization caused by the 1mg MIA injection and response to anaesthesia. As in previous chapters, these considerations and conclusion are limited by the failure to characterize the histopathology of animals used – as there can be no certainty of an OA condition driving changes in thresholds in any one rat.

Previous work has demonstrated the effect of both chronic pain conditions and anaesthesia on withdrawal thresholds and activation of RVM ON-cell firing in response to peripheral stimulation. Carlson et al 2007 demonstrates this clearly in a neuropathic model of pain. While sham animals would only exhibit EMG and ON cell responses to 60g vF and upwards, SNL animals exhibited both EMG and ON cell responses to 6g vF (Carlson, Maire et al. 2007). Similarly, PWT were significantly increased in anaesthetized animals, though the reduction in

the threshold in neuropathic pain animals remained (Carlson, Maire et al. 2007). Similar studies have demonstrated in rats 5 days after CFA injection to the paw that the magnitude of the ON cell response relative to the vF force increased during inflammation. This study also showed that the threshold for ON cell evoked responses was greater than 15g, since this force evoked no change in ON cell firing (Khasabov, Brink et al. 2012). This aligns to the fact that within this study, the greatest number of Neutral-like responses is observed following the 8g and 15g vF hairs.

However, this fails to account for the Neutral-like responses to noxious stimuli sufficient to provoke withdrawal of the paw or twitching within the leg muscles. It similarly fails to account for the OFF-like behaviours of cells that have behaved as ON-cells to noxious tail heat evoked flick. One possible explanation is that the responses of cells in the NGC, much like the RVM, are more fluid than a simple ON, OFF or Neutral designation and instead that responses may differ as a result of location or tissue type (e.g. cutaneous vs. joint vs. viscera).

This is not the first time such observations have been made. In work recording from the NGC while stimulating either the upper and lower canine teeth, radial nerve or sciatic nerve Pearl and Anderson classified a group of cells they terms M cells, or mixed cells. These cells response depended on the site of stimulation, including an ON like response to one site with an OFF like at another – though unfortunately the study does not discuss which stimulation site corresponded to which result, if a clear relationship was seen at all (Pearl and Anderson 1978). Similar such observations were made by Guilbaud et al 1973, who identified both “mixed” responder cells following IA bradykinin when recording from NGC, but additionally found that, of the 73% of 112 cells that responded to IA BK with excitation 43% did not response to noxious pinch or pressure to the paw, but did response to light touch or tapping (Guilbaud, Besson et al. 1973). As such it seems clear that while the cells of the NGC are responsive to both noxious and non-noxious stimuli, including mechanical, chemical and electrical stimulation (Casey 1969, Casey 1971, Guilbaud, Besson et al. 1973, Morrow and Casey 1983), the response profile depends on the site and type of stimulation presented.

Within the RVM, previous studies have demonstrated a clear dissociation between the responses of neurones to colorectal or bladder distension, such that classification by tail flick activity did not predict the response to distension stimulation (Chandler, Oh et al. 1994, Brink and Mason 2003, Sikandar and Dickenson 2011). Further to this, Sikandar et al 2011 observed that RVM neurones exhibited changes in activity to innocuous stimulation, specifically a 20mmHg colorectal distension, suggesting this represented the encoding of mechanical

distension by afferent capable of encoding through the range of mechanical stimulation: Innocuous through to noxious(Sikandar and Dickenson 2011).

Further back, studies have looked to compare the responses of RVM cells to differing modalities of nociceptive stimulation. Leung and Mason 1998 demonstrated that of 17 ON cells, classified by the response to heat evoked tail flick, 1 did not change firing to tail clamp; 2 were not excited by pinches to the paw; while 6 were activated by innocuous brushing of at least one site(Leung and Mason 1998). Similarly, 8 of 12 cells defined as Neutral to noxious tail heat either increased or decreased their firing in an ON or OFF like manner to tail clamp(Leung and Mason 1998). In another, the noxious stimulation of extremities including pinch of the tail, the hindpaw, the forepaw, the ear, the nose, the forehead, and to light tactile stimuli applied by gentle brushing of the cornea revealed divergent responses in 3/13 ON-cells and 11/23 OFF cells identified by their response to noxious tail heat(Ellrich, Ulucan et al. 2001). More recently Khasabov et al 2015 demonstrated that RVM cells classified by cutaneous paw pinch did not universally respond as classified to the pinching of the skin overlying the temporomandibular joint(Khasabov, Malecha et al. 2015). This suggests that certain subpopulations of RVM cells, much like NGC, behave differently depending on the somatotopic location of the cutaneous nociceptive stimulus or the modality of the stimulus. It is suggested that of these diverging responses, the differences are most common to neutral cell populations, though not exclusively(Schnell, Ulucan et al. 2002).

The suggestion of such studies is that NGC and RVM cells do not respond universally, regardless of stimulus location or nature (thermal, pinch, brush), underlining our previous understandings of the complexity of the pain control systems. It similarly explains how cells defined by noxious tail heat could behave differently to noxious and innocuous mechanical stimulation of the paw and knee, as observed here.

5.4.4 Is the MIA Model insufficiently severe to evoke a change in firing?

Comparison of the evoked responses of ON cells behaving in an ON-like manner to mechanical stimulation of the paw and knee in 1mg MIA and sham animals reveals no significant difference in the magnitude of firing. This suggests that while the injection of 1mg MIA to the left knee results in significant differences in ipsilateral and contralateral PWT, there is not a significant change in the response magnitude in the NGC. This aligns to results discussed in Chapter 4, in which no significant effect of 5HT₃ receptor antagonism is observed in either the 1mg MIA model or shams, suggesting an absence of descending serotonergic facilitation.

There is no clear consensus on the expectations of ON cell responses during pain conditions such as neuropathy or inflammation. While both Carlson et al 2007 and Silva et al 2003 failed to identify any difference in the rate of firing of ON cells in response to noxious stimulation in SNL or diabetic neuropathy rats respectively (Carlson, Mair et al. 2007), studies in inflammatory pain 4 days after CFA injection showed a significant increase in the number of impulses evoked in ON cells to both mechanical and thermal stimuli (Khasabov, Brink et al. 2012). While such differences could be attributed to different etiology of neuropathic and inflammatory pain models, other studies highlight time specific differences in neuropathic pain such that noxious pinch or CRD evoke significantly greater increases in ON cell firing in SNI animals at 8 weeks, but with no significant difference to shams at 1 week (Gonçalves, Almeida et al. 2007).

Taken together, this could suggest that such changes in evoked activity are dependent upon the extent of changes driven by the peripheral insult, likely the combination of both peripheral and central sensitization, which will also be time dependent. As such, this would attribute the significantly greater increase in ON cell evoked responses following CFA, as observed by Khasabov et al 2012, to enhanced recruitment of the RVM as a result of the extent of peripheral sensitization in inflammation. Similarly, that significant differences are only observed 8 weeks into nerve injury models may suggest that neuropathy must reach a certain severity of ascending drive. Such stimulus severity dependence for the recruitment descending controls from the RVM have previously been described (Green, Scarth et al. 2000, Peters, Hayashida et al. 2010, Bardin 2011), and are discussed in more depth in Chapter 4. Further work, using a large dose of MIA and later time points may reveal significant differences in evoked activity of ON cells versus shams. This would align with previously discussed results and conclusions, in which no significant descending serotonergic facilitation is observed in the 1mg MIA model, using spinal cord electrophysiology, while adaptive changes in descending facilitation are observed in the 2mg model (Rahman, Bauer et al. 2009).

Conversely however, the failure to characterize a shift in the response properties of the neurones recorded may instead relate to the predominance of NGC in these recordings. For example, Robinson et al 2002 argue that the differing observations of work by Pertovaara and Tukeva with those of Robinson can largely be attributed to the relative number of NGC neurones recorded (Robinson, Calejesan et al. 2002). In Robinson's RVM study, 4 recorded cells were within the NGC with the majority in the NRM – with the study identifying decreased activity of neutral cells and the awakening of previously silent cells in the second phase of the formalin model (Pertovaara and Tukeva 1989, Robinson, Calejesan et al. 2002). Conversely, Pertovaara largely recorded in the NGC and characterized no change in the firing patterns in the

bulbospinal neurones in the second phase of the Formalin model (Pertovaara and Tukeva 1989). As such it may not be that the 1mg MIA model was insufficiently severe to recruit changes in the brainstem, but instead that the NGC is less prone to adaptations in evoked and basal activity during ongoing pain.

Such electrophysiological differences between the potential response of RGC and RVM in chronic pain conditions are underlined by previously defined functional differences. Wei et al 1999, by lesioning either the NGC/NCG α or NRM, demonstrated that while lesions of the former reduced chronic inflammation induced hyperalgesia and spinal c-Fos expression, lesions of the latter increased thermal hyperalgesia and spinal c-Fos (Wei, Dubner et al. 1999). Using a NMDA receptor antagonism da Silva et al 2010 similarly identified such functional differences, in which antagonism in the RVM reversed both muscle and cutaneous hypersensitivity following repeated intramuscular injections of acidic saline, whereas antagonism in the NGC only reversed cutaneous sensitivity (Da Silva, DeSantana et al. 2010). Interestingly this suggests that NGC makes a notable contribution to the development of a cutaneous secondary hyperalgesia, potentially explaining the unexpected greater (though not significant) magnitude of change in firing to vF 15g in MIA animals (Figure 5.8 and 5.9).

Greater clarity overall, on the effect of MIA on the behaviour of the cells of the RVM and/or NGC would be gained by the study of both more cells, including the effect of MIA on OFF cells, more time points later into the MIA model, as well as naïve and 2mg MIA animals. This would allow greater understanding of the role of knee pathophysiological changes upon the RVM. At present, current data indicated that limited sensitization occurs during the 1mg MIA model, similar to conclusions in chapter 4 suggesting limited descending control.

5.4.5 Study Limitations

In considering the results and potential conclusions of this chapter, it is important to consider the limitations of the present study's design – both to provide context that will inform the conclusions, but also suggest potential modifications to the study that would have allowed greater clarity.

Evidence of Structural Pathology

As is discussed in more detail in other chapters, a major limitation in the discussion of the results presented in this chapter, as in all the chapters of this thesis, is the absence of analysis of the structural pathology of these animals. While previous literature has established the histopathological changes associated with this stage of the 1mg MIA model (Ivanavicius, Ball et al. 2007, Kelly, Dunham et al. 2012, Thakur 2012), it would be a fallacy to assume that every injection of MIA was successfully delivered. Even in a clinical setting, IA therapies commonly miss their mark (in much larger knees) without ultrasound guidance (Berkoff, Miller et al. 2012). Please refer to Section 3.4.3 for greater discussion of the limitations associated with failing to characterize the presence of OA.

Confirmation of Recording Site

As has been discussed throughout this chapter, it is likely that the recordings presented herein are of cells residing in the NGC. Though the NGC is a nucleus of interest in the descending control of pain, with many studies documenting the potential role on hyperalgesia and spinal excitability (Zhuo and Gebhart 1990, Zhuo and Gebhart 1991, Zhuo and Gebhart 1992, Zhuo and Gebhart 1997, Wei, Dubner et al. 1999, Da Silva, DeSantana et al. 2010), no confidence can be assigned to the true location of the cells recorded from given the absence of histology to conform the recording site.

Most studies recording from the brain mark the recording site at the end of the experiment with a small lesion, is made by sending current back down the electrode. The rat can then be perfused with formalin, the brain frozen, and sections cut and stained to reconstruct the recording site. The failure to do so, and instead basing location assumptions on the recording coordinates relative to bregma and lambda, means no definite conclusions can be taken. While the rat atlas and these coordinates provide a guide, the individual variability of rats by weight may leave the recording location uncertain. While it is clear the recording site contained cells responsive to nociceptive and non-noxious inputs, confident conclusions cannot be made.

5.4.6 Overall Implications

Herein I sought to investigate any adaptation to the electrophysiology of cells in the RVM during the 1mg MIA model of OA. While no confident conclusions can be taken in the absence of recording histology, it appears the majority of recordings took place in the NGC without any significant adaptations observed. I have suggested that the classifications of cells by the response based upon heat evoked tail flick may be too simplistic an understanding, as these responses may vary based upon the form and location of the stimulation. I have also postulated that the lack of difference in the evoked responses between MIA and Sham animals may suggest a lack of recruitment of descending facilitation in the MIA model, in agreement with pharmacology work presented in Chapter 4.

Chapter 6 –Peripheral Contributions to Osteoarthritis Pain

In the previous Chapters of this work, I have raised questions about the contributions of descending controls in the pain associated with osteoarthritis during the 1mg MIA model. The failure to characterize a descending drive places further emphasis on the role of the periphery in the generation of pain during joint pathology. This work aimed to use mouse lines, which had previously been characterized as having deficits in their pain or mechanosensory profiles, to identify possible sensory afferent populations or channels which may underlie the peripheral transmission of noxious mechanical stimuli or mechanical sensitization during OA and thus represent future avenues for analgesia in OA.

6.1 The Use Of Mouse Models To Identify The Contribution Of Specific Proteins In Healthy And Pathological Sensation

In much the same way that the MIA model is used as a tool to study the physiological processes underlying OA pain, transgenic mice that have been genetically manipulated and bred to lack, under express, over express *or de novo* express certain genes to allow a better understanding of the specific contributions of the encoded proteins in healthy and pathological functioning. The first use of such techniques by Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies earned the Nobel Prize for Physiology and Medicine in 2007(Manis 2007), and has since become common lab practice, representing the fast growing use of lab animals to date(HomeOffice 2013). For a full review of both the history, significance and techniques used, see Hall et al 2009(Hall, Limaye et al. 2009).

6.1.1 Voltage Gated Sodium Channels

As mention in Section 1.4.2.2, voltage gated sodium channels (VGSC) define the electrophysiological properties of primary afferents. These channels are crucial to the initiation and propagation of action potentials, and consequentially are pivotal in the transmission of peripheral sensation and pain.

The VGSC have distinct properties and locations that define both their function but also the properties of the tissues in which they reside. While specific VGSC populate cardiac tissue, namely Nav1.5 and 1.1, others are restricted to nervous tissue(Wood, Boorman et al. 2004). Nav 1.7, 1.8 and 1.9 are found throughout the DRG and have been implicated in determining pain thresholds and the development of pathological pain conditions(Momin and Wood 2008, Minett, Nassar et al. 2012).

Nav1.7, the VGSC which regulates release of peptide transmitters from central terminals and consequently wind-up, is also key to initiating action potentials following a sensory depolarizing potential, and has been demonstrated to exert a key role in ectopic firing in neuromas(Dib-Hajj, Cummins et al. 2010, Minett, Nassar et al. 2012). In studies in which the Nav1.7 gene (SCN9A) is ablated in all sensory neurones mice lose all mechanical and inflammatory pain, as well as reflex withdrawal to heat, while maintaining neuropathic pain capabilities(Minett, Nassar et al. 2012). In contrast, deletion of SCN9A in both sensory and sympathetic fibers now abolishes this neuropathic pain(Minett, Nassar et al. 2012). Interestingly, because Nav1.7 is pivotal to the conversion of a receptor potential to action potential, bridging the gap between building depolarization and the activation of Nav1.8 VGSC, the knock out of SCN9A from the subset of afferents utilizing Nav1.8 is greater than would perhaps have been expected as it essentially silences Nav1.8 too(Nassar, Stirling et al. 2004, Minett, Nassar et al. 2012). The effect of this nociceptor-specific gene deletion is a lost sensitivity to noxious grade mechanical stimuli and deficits in inflammatory pain, but maintained thermosensation(Nassar, Stirling et al. 2004, Minett, Nassar et al. 2012). Additionally, such mice showed that loss of SCN9A reduced the release of peptides at the central terminals, blocking windup(Minett, Nassar et al. 2012). This underlies the threefold role of Nav1.7 in pain sensation – to initiate the action potential by recruiting Nav1.8(Momin and Wood 2008); propagating nociceptive signals to the spinal cord; and regulating central sensitisation through control of transmitter release at central terminals(Minett, Nassar et al. 2012).

Various gain of function genetic mutations in Nav1.7 have been characterized as producing primary erythromyalgia and paroxysmal extreme pain disorder (PEPD) (Yang, Wang et al. 2004, Dib-Hajj, Rush et al. 2005, Fertleman, Baker et al. 2006), while a loss of function mutation has produced entire families with congenital insensitivity to pain (CIP)(Weiss, Pyrski et al. 2011). Similarly, SCN9A, the gene for Nav1.7, is one of only 5 genes directly linked to OA pain(Thakur, Dawes et al. 2013). As such, there is a clear clinical justification for targeting this Nav1.7 in an attempt to block the transmission of pain from the periphery – a pharmacological target experiencing mixed success in clinical trials at present, with success in trigeminal neuralgia(Zakrzewska, Palmer et al. 2013) but recent failures in OA pain(Ltd. 2015).

Nav1.8 is also understood to play a crucial role in nociception and pain conditions. Nav1.8 is a tetrodotoxin (TTX) resistant VGSC which has an all-or-nothing role in action potential generation in sensory afferents(Liu and Wood 2011), through the action potential upstroke, and exhibits the rare ability to transmit sensory information to the CNS at cold temperatures, unlike the other 8 VGSCs(Zimmermann, Leffler et al. 2007, Liu and Wood 2011).

Crucially, this VGSC has a restricted expression compared to Nav1.7, with Nav1.7 found throughout sensory and sympathetic fibers while Nav1.8 is restricted to a subset of sensory afferents. Specifically, 75% of DRG cells from L4-5 express Nav1.8, of which this includes the majority of the nociceptive afferents serving this area (~90%, accounting for 60% of the total Nav1.8+ population)(Shields, Ahn et al. 2012). Beyond the nociceptors, Nav1.8 is also expressed in the C-low threshold mechanosensors as well as rapidly adapting A β low threshold mechanosensory afferents. In other words, Nav1.8 underlies the electrical activity of multiple sensory modalities, both noxious and innocuous, notably mechanical and cold.

Nav1.8 knock out mice demonstrate a loss of noxious mechanical sensation, impaired but not lost noxious thermoreception and delayed development of inflammatory hyperalgesia(Akopian, Souslova et al. 1999), however mice maintained normal sensorimotor coordination and otherwise appeared healthy, though a counterbalancing increase in Nav1.7 expression was observed. Further studies similarly showed loss of cold sensitivity and mechanosensation during the cold(Zimmermann, Leffler et al. 2007). Such work highlighted the importance of Nav1.8 to mechanical hyperalgesia, pain sensation during cold and cold pain, as well as development of inflammatory pain. The latter is the result of the fact Nav1.8 becomes heavily phosphorylated by inflammatory mediators, causing a potentiation of the ion current and altered expression, both in terms of protein levels and distribution(Vijayaragavan, Boutjdir et al. 2004).

Crucially for the investigation of mechanisms underlying OA pain, these studies have outlined the importance of Nav1.8 in the transmission of noxious mechanosensation and development of inflammatory pain. As such, Nav1.8 is a logical candidate in the development of OA pain therapeutics. The first part of this study is looking to characterize the MIA pain profile of a mouse line previously described in Abrahamsen et al 2008, the DTA mouse(Abrahamsen, Zhao et al. 2008). In this mouse line all the post-mitotic sensory neurones containing Nav1.8 have died through the expression of diphtheria toxin A (DTA)(Abrahamsen, Zhao et al. 2008). These DTA mice were generated by crossing heterozygous Nav1.8 knock-in Cre-expressing mice with homozygous DTA floxed mice, to produce equal numbers of control mice (wild type, WT) and DTA expressing mice, whose Nav1.8 containing neurones die as a result of toxicity of DTA. Similarly to mice where Nav1.7 is knocked out of Nav1.8 containing neurones, these mice exhibit a loss of sensitivity to noxious mechanical, cold and inflammatory pain(Abrahamsen, Zhao et al. 2008). Herein I have tested the hypothesis that such mice, which lack noxious mechanical and inflammatory pain, would be resistant to the development of OA like pain from the MIA model.

6.1.2 Transient Receptor Potential Channels

The microarray data for the DRG cells of the DTA mice show a significant loss of TRP family receptors, notably TRPC6 and TRPC3, as a result of the ablation of the Nav1.8 containing neurones (Abrahamsen, Zhao et al. 2008). Given differences observed between wild type (WT) and DTA mice in pain behavioural profiles, the TRPC family was proposed as a possible contributor to these differences in mechanosensation.

The Transient Receptor Potential (TRP) channels are a family of non-selective cation channels with roles across olfaction, taste, chemosensation, thermosensation and mechanosensation. As discussed in Section 1.4.2.1, these 6 transmembrane structures form pores by associating into homo or heterotetrameric structures (Christensen and Corey 2007). The TRPC (canonical) family have been implicated heavily in the detection of mechanical stimuli, both through direct membrane stretch activation and through second messenger systems, largely through the conversion of PIP₂ to DAG in the neighbouring membrane (Spasova, Hewavitharana et al. 2006). TRPC6 is found throughout cell populations responding to hydrostatic pressure changes, such as podocytes in the glomerulus and vascular smooth muscle, implicating them in pressure regulated processes (Spasova, Hewavitharana et al. 2006, Quick, Zhao et al. 2012). TRPC3 on the other hand has only recently been implicated in mechanosensation – where TRPC6^{-/-} mice fail to show any behavioural deficits in mechanosensation they do display elevated blood pressure, linked to a compensatory overexpression of TRPC3 (Spasova, Hewavitharana et al. 2006). Similarly, the TRPC3^{-/-} mouse shows no behavioural deficits, but the DKO mouse show a consistent loss in sensitivity to innocuous punctate stimuli (Quick, Zhao et al. 2012). Both TRPC3 and TRPC6 are expressed in small diameter sensory neurons of the DRG (Elg, Marmigere et al. 2007), where these channels have previously been shown to associate (Goel, Sinkins et al. 2002).

Previous work investigating the possible role of these TRPC channels has shown that the transcriptionally regulated, slowly-adapting mechanosensitive current observed in this population of Nav1.8⁺ neurones is regulated by TRPC channels, as confirmed by a TRPC3 and TRPC6 double knock out (DKO) mouse (Quick, Zhao et al. 2012). These DKO mice show a consistent loss of sensitivity to innocuous mechanical pressure, while maintaining normal thermal and noxious mechanical sensation. In these mice ~50% of the rapidly adapting mechanosensitive currents in small diameter cells were silenced (Quick, Zhao et al. 2012), where C-LTMs have been shown to be necessary for mechanical allodynia during inflammation, nerve injury and trauma previously (Seal, Wang et al. 2009).

Given the important role of sensitized mechanosensory systems in OA pain, these channels represent an interesting candidate in the search for mechanosensory machinery whose sensitization may

underlies the development of mechanical hypersensitivities during OA. Herein I have tested the hypothesis that such mice, either DKO for TRPC3 and TRPC6, or SKO for one of either TRCP3 or TRPC6 may exhibit differences in the mechanosensitive profiles observed during the MIA model of OA pain, compared to WT mice.

6.1.3 Chapter Aims

For patients with symptomatic OA the unmet needs are still clear – efficacious, safe and tolerable analgesia. If we are to address these to provide superior quality of life for patients, it is crucial that we continue to expand our knowledge of the mechanisms underlying pain during OA so we can better manipulate and target these mechanisms to mitigate their impact.

The purpose of the work presented in this chapter was to confirm whether: first, the subpopulation characterized by the expression of Nav1.8 were a requirement for the expression of pain in osteoarthritis, and thus whether Nav1.8 blockade would represent a viable target for the management of OA pain; and second, whether TRPC3 and TRPC6 channels contribute to the development of mechanical hypersensitivity during OA, and thus themselves may similarly act as a viable target for analgesia in OA. By understanding the contributions of these specific receptors and neuron populations, there is hope of defining new and more specific targets for the management of OA pain.

6.2 Methods

NB: This project was performed in partnership with Michael Minett and Jane Sexton, post-doc and PhD candidates respectively in the John Wood lab at UCL.

6.2.1 Animals

All work was conducted in male and female transgenic mice and their WT littermates, bred and housed in the Cruciform Biological Services unit at University College London. As described in Chapter 2, behaviour was conducted over the 21 days of the MIA model in the following mouse lines:

- “DTA Mice”: This mouse line was generated by crossing heterozygous Nav1.8 Cre mice with homozygous eGFP-DTA mice. This generated a litter of half controls (wild type, WT) and half DTA mice, where DTA mice have all the post-mitotic sensory neurons containing Nav1.8 eradicated through the expression of diphtheria toxin A (Ivanova, Signore et al. 2005, Stirling, Forlani et al. 2005, Abrahamsen, Zhao et al. 2008). A full sensory profile is described in Abrahamsen et al 2008.
- TRPC knock out mice: Quick et al 2012 generated three mouse lines for use (Quick, Zhao et al. 2012):
 - TRPC3 SKO
 - TRPC6 SKO
 - TRPC3/6 DKO

A double knock out mouse had originally been generated by Birnbaumer and colleagues at the NIEHS. Their DKO mice were crossed with C57BL/6 mice to create heterozygous TRPC3^{+/-};TRPC6^{+/-} mice. These could then be crossed together to create DKOs, single KOs and WT controls. A full sensory profile is described by Quick et al.

6.2.2 Induction of the model

As detailed in Section 2.2, OA was induced using 0.5mg monosodium iodoacetate in 5µl of 0.9% saline using a 30G needle. Mice were aged 6-8 weeks, weighing between 20-35g depending on gender and mouse strain.

6.2.3 Behavioural Assessment

Mice were assessed on day 0, on the morning prior to injection, and on the mornings of day 3, 7, 14 and 21 there after. If, at any time point, mice displayed obvious pain or physical damage relating to fighting or over-scratching (as appeared in a minority of D mice) these animals were excluded.

As described in Section 2.3, before measurements began, all mice were given a period of 1hr in which to acclimatize to their new settings. The PWT was then assessed using the “up-down method”, as described by Chaplan et al(Chaplan, Bach et al. 1994). In brief, vF hairs of sequential increasing or decreasing force are applied, based on the response to the previous stimuli (withdrawal or lack there of). The statistical formula described by Dixon et al is then utilized to calculate the 50% withdrawal threshold(Dixon 1980) – which describes the force at which the animal will withdraw 50% of the time.

Following the assessment of PWT, incapacitance was assessed as described in Section 2.3.

6.2.4 Data Analysis

All data is presented as the mean \pm SEM.

- Punctate mechanical hypersensitivity: Data expressed as the 50% paw withdrawal threshold. Differences between transgenic mouse groups were analysed using a two way ANOVA followed by Bonferroni post-hoc tests.
- Incapacitance: Data expressed as the percentage of total weight borne on the ipsilateral side. Differences between transgenic mouse groups were analysed using a two way ANOVA followed by Bonferroni post-hoc tests.

Values were deemed significant at $p < 0.05$.

6.3 Results

6.3.1 Behavioural Hypersensitivity in the MIA model in WT and DTA mice

Punctate mechanical hypersensitivity and incapacitance was assessed in DTA mice and their WT littermates following the intra-articular injection of 0.5mg MIA at days 0, 3, 7, 14 and 21. These tests revealed the gradual development of a significant punctate mechanical hypersensitivity and reduction in percentage weight borne on the ipsilateral paw over time in WT mice. While the effects of time were similarly significant in the DTA mice, the behavioural profiles following MIA were not identical to those of the WT mice.

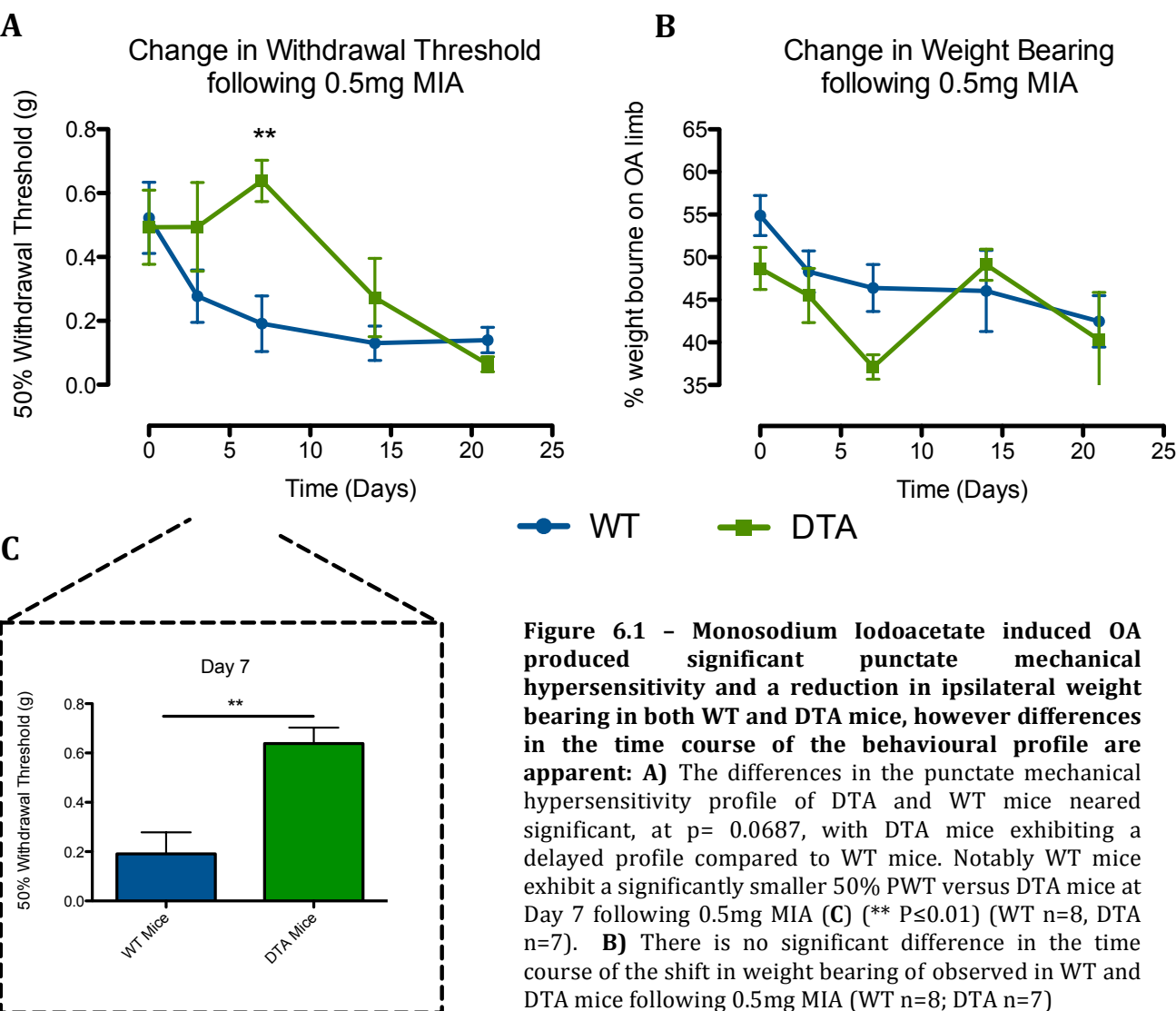


Figure 6.1 - Monosodium Iodoacetate induced OA produced significant punctate mechanical hypersensitivity and a reduction in ipsilateral weight bearing in both WT and DTA mice, however differences in the time course of the behavioural profile are apparent: A) The differences in the punctate mechanical hypersensitivity profile of DTA and WT mice neared significant, at $p = 0.0687$, with DTA mice exhibiting a delayed profile compared to WT mice. Notably WT mice exhibit a significantly smaller 50% PWT versus DTA mice at Day 7 following 0.5mg MIA (**C**) (** $P \leq 0.01$) (WT $n=8$, DTA $n=7$). **B)** There is no significant difference in the time course of the shift in weight bearing of observed in WT and DTA mice following 0.5mg MIA (WT $n=8$; DTA $n=7$)

While the effect of time was highly significant in both WT and DTA Mice (Figure 6.1A; 2-Way ANOVA; $p < 0.0001$), indicating a significant decline in PWT following MIA injection in both animals, a near significant difference is observed in the time course of the developing hypersensitivity between these mouse groups, at $p = 0.0687$. This difference reflects the delayed time course of decreasing 50% PWT in the DTA mice versus WT, with the greatest effect of this delay observed at day 7 (Figure 6.1C; Bonferroni posttests; $** P \leq 0.01$). This delayed punctate mechanical hypersensitivity in DTA mice is temporary, with very similar 50% PWT of $0.14 \pm 0.04\text{g}$ and $0.06 \pm 0.02\text{g}$ for WT and DTA mice respectively, demonstrating a strong, similar mechanical hypersensitivity in both lines by this time point.

Though a significant effect of time was observed for the weight borne on the ipsilateral paw following MIA in WT and DTA mice, no significant difference was observed between the two mouse lines, with both showing a maximum change to any given time point of roughly $\sim 10\%$ (Figure 6.1B; 2-way ANOVA). However, the differences in time course of the change is interesting, given the WT mice show a gradual increase in incapacitance to day 21 while the DTA mice exhibit the greatest incapacitance at day 7, with a slight recovery there after.

As such, DTA mice appear to show no difference in incapacitance behaviour to WT mice, however there is an apparent delay in the time course of punctate mechanical hypersensitivity, which may reflect the protective effects of loss of Nav1.8 containing neurones.

6.3.2 Behavioural hypersensitivity during the MIA model in WT, SKO and DKO mice

Punctate mechanical hypersensitivity and incapacitance was assessed in TRPC3 SKO, TRPC6 SKO, their WT littermates and TRPC3/6 DKO mice over the 21 days following injection of 0.5mg MIA. A significant effect of time was observed across all groups to both behavioural measures, indicating the development of significant mechanical hypersensitivity and shifts in weight bearing following 0.5mg MIA. However no significant difference was observed between these mouse groups (Figure 6.2; 2-Way ANOVA).

All mice exhibited a rapid decline in the 50% PWT to day 3, plateauing across all groups between 50% PWTs of roughly 0.1 and 0.25g from days 7 to 21 (Figure 6.2A). No differences were detected either statistically or are visually apparent. Similarly, the shape of the time course of change for these mice is broadly similar to those observed in the DTA mice's WT littermates in Figure 6.1.

A broadly similar time course in the shift of weight bearing following injection of MIA is similarly observed across these four groups of mice (Figure 6.2). Mice exhibit a rapid shift in weight bearing of

roughly 10%, seen in SKO and DKO mice to Day 3 and slightly slower to develop in WT mice to Day 7, followed by a rebounding of weight bearing by day 14 onwards, reflecting the development of a sensitivity in the knees which recovers after this initial early flare in the first week. No significant differences between these four groups of mice were observed.

As such these SKO and DKO mice appear to show no difference in the behavioural hypersensitivity profile versus WT mice during the MIA model of OA, indicating that the loss of TRPC3 and/or TRPC6 dose not protect against OA pain.

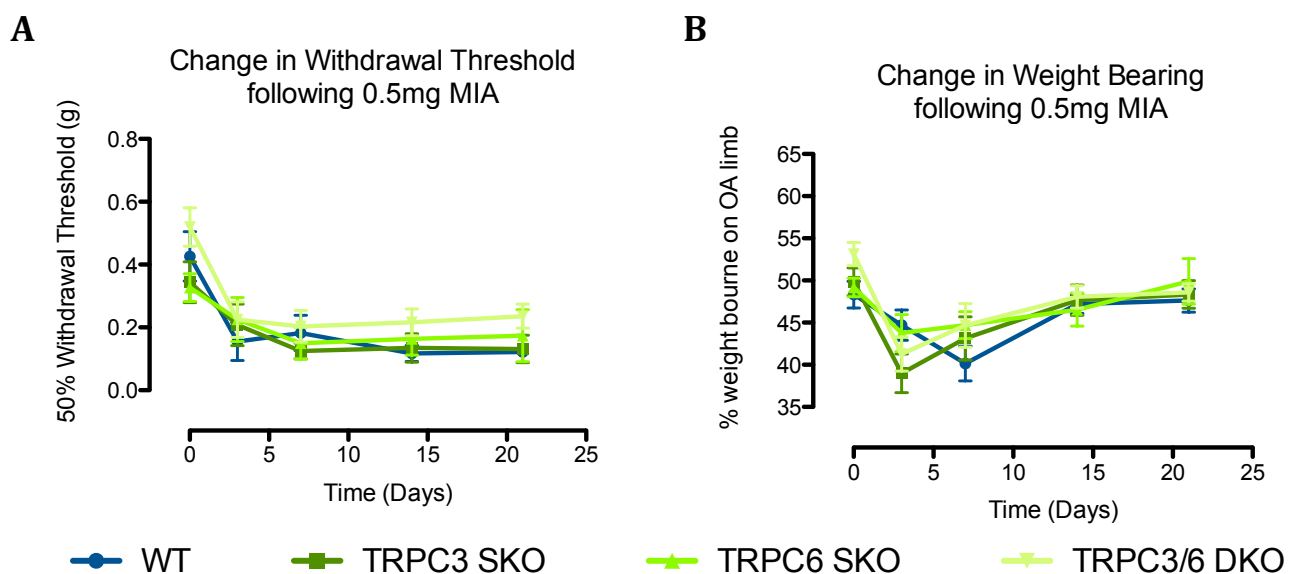


Figure 6.2 - Monosodium Iodoacetate induced OA produced significant punctate mechanical hypersensitivity and a reduction in ipsilateral weight bearing in both WT, TRPC3 SKO, TRPC6 SKO and TRPC3/6 DKO mice, with no significant differences in the behavioural profiles of these four mouse lines. A) All four mouse lines exhibit a rapid decline in the 50% PWT to Day 3 and 7, which is maintained to day 21 following 0.5mg MIA (WT n=10; TRPC3 SKO n=10; TRPC6 SKO n=10; TRPC3/6 DKO n=10). **B)** All four mouse lines exhibit a rapid decline in weight borne on the ipsilateral limb in the 3-7days following 0.5mg MIA injection, but rebound to near original distribution of weight to days14 and 21. MIA (WT n=10; TRPC3 SKO n=10; TRPC6 SKO n=10; TRPC3/6 DKO n=10)

6.4 Discussion

The use of transgenic mice has enabled the characterization of the roles of specific proteins in healthy somatosensation and pathological pain conditions. Previous work has established, through the generation of a number of different mouse lines, both the role of Nav1.8 containing neurones in noxious mechanosensation and inflammatory pain, and for TRPC3 and TRPC6 in innocuous mechanical pressure.

The work presented here has demonstrated that while the ablation of Nav1.8 containing neurones may protect against the initial stages of the MIA model in these DTA mice, this does not provide protection from the punctate mechanical sensitivity which develops in WT MIA mice in the later days of the model. This work has similarly demonstrated that while TRPC3 and TRPC6 may have a role in innocuous pressure sensation, these channels do not play a part in the punctate mechanical threshold or weight bearing changes observed during this model of OA pain.

6.4.1 Behavioural hypersensitivity in a murine model of MIA induced OA pain

As discussed in Section 3.4.1, behavioural hypersensitivity following a dose of MIA to the knee is well characterized as including both shifts in weight bearing and punctate mechanical hypersensitivity in the rat (Bove 2003, Fernihough, Gentry et al. 2004, Pomonis, Boulet et al. 2005, Rahman, Bauer et al. 2009, Vonsy, Ghandehari et al. 2009, Sagar, Staniaszek et al. 2010, Kelly, Dunham et al. 2012, Thakur, Rahman et al. 2012, Burnham and Dickenson 2013). Similar efforts have been made to characterize these changes in mice, given the opportunity transgenic mice offer to the study of OA pain. However, the translation of this model to mice is difficult to scale. The smaller size of mice raise questions about injection volume, needle gauge and most importantly, the dose of MIA to be used – not least because some of the doses used in rats, namely >1mg, have been lethal in mice (unpublished – known experience of several UK labs, including Nottingham, UCL and KCL). As a result, the dose used in the MIA model in mice has varied broadly, from 0.025 to 1mg per knee, in volumes between 5-10µl (van der Kraan, Vitters et al. 1989, Van Osch, Van Der Kraan et al. 1994, Harvey and Dickenson 2009, Ogbonna, Clark et al. 2012, Bowles, Mata et al. 2014). However in those studies that tested pain-like behaviours, using the upper and lower doses described, all doses induced significant mechanical threshold and weight bearing changes (Harvey and Dickenson 2009, Ogbonna, Clark et al. 2012, Bowles, Mata et al. 2014, Horváth, Tékus et al. 2016).

In line with these prior studies, this work presented in Figure 6.1A and 6.2A demonstrates that the injection of a 0.5mg dose of MIA to WT mice results in a rapid decline and plateau in the 50% paw

withdrawal threshold to roughly 0.2g. In Ogbonna et al 2013, where one of three doses investigated included the same 0.5mg dose, a very similar decline and plateau is observed, through the extent of the reduction in PWT is greater – at <0.1g by day 14(Ogbonna, Clark et al. 2012).

Slightly less aligned are the effects of MIA on the percentage weight borne on the ipsilateral paw. While in Figure 6.1B the WT mice exhibit a slow but steady shift in the weight borne by roughly 10% to the final day, the mice in Figure 6.2B decline more rapidly to days 3-7, by a similar 10%, but then regain to settle at a less severe shift. This suggest the development of a hypersensitivity at the knee in both mouse strains, suggesting a pain/discomfort at rest that may relate to increased spontaneous C fibre activity (Kelly, Dunham et al. 2012) (As discussed in Section 3.4.1). However the time courses differ. Interestingly, the DTA mice themselves show a weight distribution profile over time which is far more similar to that seen in mice in Figure 6.2B. Such differences in weight distribution profiles may be an artefact of the mouse strains, since the WT mice in Figure 6.1B are different from those in Figure 6.2B – where previous work has identified differences in pain profiles between different mouse or rat strains(Mogil, Wilson et al. 1999, Yoon, Lee et al. 1999, Lovell, Stuesse et al. 2000, Felice, Sanoja et al. 2011).

Previous murine OA studies have characterised a “recovery” profile of incapacitance in the MIA model, in which the mice recovered back to the baseline weight distribution observed in comparator animals(Bowles, Mata et al. 2014, Horváth, Tékus et al. 2016). In one of the studies this was explained by the author as a likely artefact of the low dose, given just 0.05mg of MIA was used to model OA pain(Bowles, Mata et al. 2014) . The initial drop in weight bearing was attributed to inflammation, which recovered; where a chronic shift might eventually have been seen as pathology in the knee progressed(Bowles, Mata et al. 2014). However, Horvath’s work using the same dose as used in this chapter also reported a recovery in the shift in weight distribution by day 14 in WT mice, similar to that observed in figures 6.2B(Horváth, Tékus et al. 2016). It is worth noting however that a recovery profile is not seen in high dose models like the 1mg murine model used by Ogbonna et al(Ogbonna, Clark et al. 2012). The shift reported in Ogbonna et al 2012 is maintained up to the 28th day of observation, much like the profiles reported in rats by Bove et al 2003(Bove 2003). As such, it may be that across all the doses of MIA used a change to the weight bearing profile, indicative of primary hypersensitivity, is observed in the initial~10days, but only in the larger 1mg dose is this maintained. It could be suggested that the initial shift in weight bourne, across all doses, could be attributed to an early inflammation, and at latter time points (14days onwards) is driven by a more chronic pathology at the joint that is potentially more pronounced in the higher dose model.

While these suggested explanations are limited by the failure to characterise inflammation, such as synovitis or joint volume, in the work presented here (and these limitations will be discussed in more

detail in 6.4.3), other studies in the mouse model of MIA have investigated these characters of the model to support the proposal. Recent work by Horvath et al has characterised a significant increase in both the mediolateral and anteroposterior knee diameter, most notably in the first 3 days of a 0.5mg MIA model(Horváth, Tékus et al. 2016). This study went so far as to call the inflammation “remarkable oedema”. Others have captured similar changes, with early work by van der Kraan capturing significant swelling in the initial days of the model(van der Kraan, Vitters et al. 1989), and Uchimura suggesting that a degree of synovitis persists to day 10 even with a much lower dose of MIA(Uchimura, Foote et al. 2016). Meanwhile Ogonna et al suggest that, given the absence of an ATF-3 profile at day 10 in their 1mg MIA mouse model, that the microgliosis observed at day 7 is driven by inflammation instead of neuronal damage at this time(Ogonna, Clark et al. 2012). While this is not as large a body of evidence as is available in the rat MIA model, this certainly supports the potential of an initial inflammatory phase driving sensitization in the first week after MIA injection.

In contrast to the recovery observed in WB, the decline in PWT, which occurs in the first 3-7 days, is maintained instead. This suggests that secondary sensitization, indicative of the development of central sensitization, does not recover after this postulated initial inflammatory stage. It seems plausible that this difference may be a result of the size of the MIA dose selected in this protocol, such that joint pathology may already be sufficient at Day 7-14 to maintain central sensitization. It is certainly the case that in both mouse models or rat models using larger doses, $\geq 0.5\text{mg}$ or $\geq 2\text{mg}$ respectively, the changes in 50% PWT plateau across a time course(Combe, Bramwell et al. 2004, Ogonna, Clark et al. 2012). That larger doses could institute changes of this severity of joint pathology so rapidly can be understood from the comparative time course and locations of lost proteoglycan synthesis, where larger doses have a significant effect across both central and peripheral patella cartilage, with the greatest effect in the first week, while lower doses fail to effect peripheral cartilage (Guingamp, Gegout-Pottie et al. 1997).

However, given the hypothesis that the $>2\text{mg}$ dose in rats triggers neuropathic mechanisms in the MIA model, it is also valid to question at what scaled down dose this “tipping point” occurs in the mice. It is plausible that this may have happened already in mice at 0.5mg, not least given the systemic lethality of the 1mg dose in some mice. While not on a scale equivalent to the PNL model, it has been shown that 1mg MIA in mice results in a significant increase in the expression of activated ATF-3, with the author concluding that the possibility of axonal injury could not be excluded in these MIA mice – though it was felt that inflammation was more likely the underlying driver(Ogonna, Clark et al. 2012). This aligns to observation of axonal injury and neuropathic components to high dose MIA induced hypersensitivities in rats(Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012). As such, there is a possibility that the behavioural hypersensitivity exhibited in these mice, at least beyond day 7 of this model, may in part be attributed to axonal damage by the large dose of MIA escaping the

synovial joint and affecting local sensory afferents terminals – though this has not been verified in these animals. Further work to clarify this would provide valuable insight into potential explanations for this maintained decline in PWT.

6.4.2 Preserved punctate hypersensitivity at day 14 and onwards after 0.5mg MIA in mice lacking Nav1.8 neurones

The data presented in Figure 6.1A, in which near significant differences in the time course of changes in 50% PWT threshold are observed between WT and DTA mice following IA injection of MIA ($p=0.0687$, Figure 6.1A), suggests that the loss of Nav1.8 containing neurones provides protection from behavioural hypersensitivity in the paw in the first but not later weeks of this OA pain model.

It has previously been demonstrated that the loss of Nav1.8 containing neurones protects against the development of inflammatory pain but not neuropathic pain associated with the SNL, oxaliplatin and cancer induced bone pain models (Abrahamsen, Zhao et al. 2008, Minett, Falk et al. 2014). In light of this, the results presented here could suggest a potential timeline of pain mechanisms following IA injection of MIA. Namely that in the first 7 days, where a significant resistance to hypersensitivity is observed in DTA mice, pain is driven by the previously discussed inflammation, where the loss of Nav1.8 containing neurones provides protection from punctate mechanical sensitivity provided in the DTA mice. However, beyond this point, the resurgence of the punctate mechanical sensitivity in the DTA mice suggests that the pain mechanisms have evolved beyond inflammation. Given the profile of DTA mice similarly include deficits in noxious mechanical sensation (Abrahamsen, Zhao et al. 2008), it is possible to interpret these results as suggestive of neuropathic mechanisms at these later time points. Unfortunately this work did not seek to classify this (and these limitations will be discussed in more detail in 6.4.3)

As discussed earlier, previous work in mice following the injection of 1mg MIA have characterized changes that could reflect neuropathic mechanisms (Ogbonna, Clark et al. 2012). Such changes are similarly characterized in rats at doses of 2mg MIA and upwards (Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012). As such, the *possibility* of neuropathic changes during the MIA model of OA pain is established. The pertinent questions are whether the rebounding punctate mechanical sensitivity in these DTA mice is a genuine indicator of neuropathy.

The first question is addressed by our understanding of the role of Nav1.8 containing neurones in differing pain conditions, both inflammatory and neuropathic. Nav1.8 is distinct from other sodium channels in two major ways: this channel is the only channel which functions at cold

temperatures(Zimmermann, Leffler et al. 2007), but crucially this channel is the only channel whose expression is restricted to a subpopulation of sensory fibers, namely nociceptors, C-LTMs and A-LTMs(Shields, Ahn et al. 2012). Nav1.8 works in concert with Nav1.7 channels expressed in this same population, where Nav1.7 bridges the gap between building depolarization and the activation of Nav1.8 for neuronal transmission. By interrupting the activity of these neurones, through various genetic and pharmacological techniques, attempts have been made to classify a specialized population of sensory afferents. However, whether Nav1.8 and thus this population have a role in the generation of pain during neuropathy is not entirely clear-cut.

Initial work using Nav1.8 knock out mice revealed no difference in the change in mechanical withdrawal threshold of KO mice and WT mice following peripheral nerve injury(Kerr, Souslova et al. 2001), which was corroborated in later studies(Nassar, Levato et al. 2005). This suggests that this Nav1.8 containing fiber population is not required for the generation or maintenance of neuropathic pain. However, given the expression of Nav1.7 in these fibers, it is conceivable that Nav1.7 compensates for the loss of Nav1.8 to maintain the response of this fiber population to neuropathic pain, especially given Nav1.7 expression is increased in Nav1.8 KO mice(Akopian, Souslova et al. 1999). That this Nav1.8 containing population of sensory afferents is neither a requirement nor a major contributor to neuropathic pain was further suggested by work in which both Nav1.7 was knock out from the Nav1.8 positive fibers, in addition to the knock out of Nav1.8. This double KO mouse developed mechanical allodynia following SNL that was indistinguishable from either WT or Nav1.8 single KO mice(Nassar, Levato et al. 2005). Later work has similarly demonstrated that Nav1.8 KO mice develop SNT and CCI induced mechanical allodynia normally(Minett, Falk et al. 2014), while DTA mice develop SNL, oxaliplatin and cancer induced bone pain mechanical and cold allodynia to both the same degree and time course as WT mice(Abrahamsen, Zhao et al. 2008, Minett, Falk et al. 2014). The overarching suggestion of such studies is that peripheral neuropathic pain can develop independently of the Nav1.8 containing population of sensory afferents.

However, it is important to consider the body of work that contradicts this, citing the importance of Nav1.8 as a target for the management of neuropathic pain – and thus the role of this neuronal subpopulation. Changes in Nav1.8 expression patterns and sensitivity during neuropathic pain have been described, where these changes to the Nav1.8 channel population are described as enabling the development of neuropathic pain(Gold MS 2003, Thakor, Lin et al. 2009). Further, treatment with anti-sense oligonucleotides and selective antagonists such as A803467 were shown to prevent or reverse hypersensitivities caused by peripheral nerve damage(Lai, Gold et al. 2002, Gold MS 2003, Jarvis, Honore et al. 2007). That targeting Nav1.8 could provide such clear reversal of hypersensitivity counters the suggestion that Nav1.8 containing neurones are not required for neuropathic pain. However, it is plausible that both antisense and Nav1.8 antagonists could be having off target effects

that underlie this analgesia. It is similarly possible that the persistence of neuropathic pain in mice lacking Nav1.8 populations may be an artifact of the broad redundancy in sensation and nociceptive systems – such that in the absence of Nav1.8 other systems make up the difference, while in normal animals Nav1.8 contributes both through redistribution and phosphorylation. This would certainly explain the ability of Nav1.8 antagonism to reduce mechanically evoked responses in spinal cord electrophysiology in MIA but not sham animals (Rahman and Dickenson 2015). Considering these two possibilities, it seems more likely that counterbalancing adaptations in other ion channel systems may allow neuropathy to proceed uninterrupted in DTA animals. As such, the punctate hypersensitivity observed at day 14 onwards in the DTA mice may be driven by peripheral nerve damage.

The alternative explanation is that joint pathology by this time point is severe enough that it is driving central sensitization and the consequential expansion of receptive fields and referral of pain associated (Bajaj, Graven-Nielsen et al. 2001). However, this theory is reliant on an ongoing barrage of mechanical nociception from the joint (Schaible, Schmidt et al. 1987), which is understood to be absent in DTA mice, which lack noxious mechanosensation (Abrahamsen, Zhao et al. 2008). This absence and disruption of noxious mechanosensation is similarly observed in Nav1.8 KO mice (Akopian, Souslova et al. 1999, Nassar, Levato et al. 2005), mice in which Nav1.7 have been deleted from all Nav1.8 expressing neurones (Minett, Nassar et al. 2012), and double KO mice where Nav1.8 are additionally deleted (Nassar, Levato et al. 2005). Further more, as previously discussed, the results of Figure 6.1 and 6.2B suggest that at this time point a recovery from previous shifts in weight bearing has occurred – suggestive in itself that the joint is less likely to be causing pain/discomfort at rest, potentially interpreted as a recovery from increased spontaneous C fibre activity (Kelly, Dunham et al. 2012).

We must similarly consider that the ablation of this population of Nav1.8 expressing neurones will have dramatically reduced innervation of the knee joint. We know that approximately 80% of afferents serving the joint are unmyelinated (Grubb 2004), where previous work has demonstrated that up to 90% of these would be Nav1.8 containing neurones (Shields, Ahn et al. 2012). We similarly know that roughly 50% of joint afferents are CGRP positive neurones (Edoff, Grenegard et al. 2000), where CGRP expression is increased during painful OA (Fernihough, Gentry et al. 2005) – however, only 12% of CGRP containing fibers were spared in the DTA mouse (Abrahamsen, Zhao et al. 2008). This suggests that not only is noxious mechanosensation undermined in these animals, but there would be a considerable loss of input from the joint regardless of the extent of pathophysiology. It is questionable if, even in the presence of inflammatory sensitization or noxious mechanical stimuli, this restricted population of remaining afferents could provoke equivalent behavioural sensitivity to that seen in WT mice, suggesting that chronic joint pathophysiology alone is not the only force driving central sensitization.

While these considerations suggest to me that the drop in the 50% withdrawal threshold observed in DTA mice at days 14 onwards of this 0.5mg MIA murine model could, at least in part, be driven by neuropathic mechanisms I have no conclusive evidence of neuropathy in the murine MIA model of osteoarthritis. This remains one potential explanation of a behavioural profile that is difficult to explain. In addition to further work in the mouse MIA model to characterize neuropathy, such as characterization of intra-epidermal nerve fibre density in plantar hindpaw skin, or spinal cord dorsal and ventral horn microgliosis, the use of another model of OA may be considered in this mouse line to compare the behavioural profile, such as an anterior cruciate ligament transection. This would eliminate the possibility of neurotoxicity from MIA and if the DTA mice go on to develop a punctate mechanical sensitivity following this model, potentially over a similar or slower time course, a more confident conclusion could be drawn about the feasibility of Nav1.8 blockade to treat OA pain. The results presented within do not suggest Nav1.8 blockade would be successful in OA pain without inflammation/synovitis, though pharmacology work in previous MIA rat studies have suggested otherwise (Schuelert and McDougall 2012).

The lack of a significant difference between the ipsilateral weight bearing in DTA and WT mice (Figure 6.1B) suggests that, even in the absence of the Nav1.8 containing population of sensory afferents, DTA mice experience discomfort from the knee joint sufficient to shift their weight to the contralateral paw. As discussed, we would expect a drastic reduction in the nociceptors population serving the joint in these DTA mice. However, these DTA mice would still retain a large proportion of the roughly 20% myelinated fibers serving the joint whose sensitization and chronic activation could be sufficient to encourage a shift in weight bearing, reflecting perhaps more a discomfort than ongoing pain. These raise important questions however about the extent and nature of the remaining innervation of the joint in DTA mice, since it is clear that the loss of Nav1.8 containing neurones is not sufficient to alleviate the pain or discomfort motivating shifts in weight bearing.

6.4.3 The behavioural profile of TRPC3 and TRPC6 SKO and DKO mice

By both measures, punctate mechanical sensitivity and weight distribution, the KO of either TRPC3 or TRPC6 or both fails to affect the behavioural profile evoked by the IA injection of 0.5mg MIA. This suggests that these SKO and DKO mice have either developed a counterbalancing increase in another mechanosensitive receptor to compensate for these deletions, or that TRPC3 and TRPC6 are not involved in the mechanosensitization observed in mice following in the injection of MIA into the IA space.

The original hypothesis was that these mechanoreceptors might be involved in the generation of inflammation related mechanical sensitization, becoming sensitized during the early phase of the MIA model to underlie primary hypersensitivity reflected in weight bearing changes. This hypothesis was driven by understandings from previous studies, which suggested both the localization and function of these receptors could lend themselves to mechanical sensation and allodynia.

During the study of the DTA mice by Abrahamsen et al 2008, it was demonstrated that TRPC3 and TRPC6 expression in the DRG was significantly diminished in DTA mice versus WT littermates, suggesting these ion channels may be most commonly expressed in small diameter sensory afferents(Abrahamsen, Zhao et al. 2008). This is corroborated by work using real time PCR to examine the presence of TRPC1-C7 in DRG, which characterised the expression of TRPC3 in roughly 30% of the DRG in exclusively in the non-peptidergic IB4+ TRPV1- small diameter afferents(Elg, Marmigere et al. 2007). This study similarly characterised TRPC3 and TRPC6 as the most abundant of this family of receptors.

A range of work had similarly characterised a role for TRPC channels in mechanosensation. While the impact is lesser in SKO mice, due to corresponding compensatory increases in related TRPC receptors(Spassova, Hewavitharana et al. 2006), DKO mice exhibited selective deficits in touch response and cultured DRGs electrophysiology revealed deficits in mechanotransduction(Quick, Zhao et al. 2012). These channels, in concert with other TRPs through the formation of heteromeric calcium channels, have similarly been identified for their roles in the development of mechanical hypersensitivity and nociceptors sensitization during inflammation(Alessandri-Haber, Dina et al. 2009, Ding, Xiao et al. 2011), leading to speculation that this family of TRPC channels may contribute to mechanical allodynia during disease models such as OA(Eijkelkamp, Linley et al. 2013, Lolignier, Eijkelkamp et al. 2015), especially in light of the sensory profile and inflammatory pain deficits in DTA mice(Abrahamsen, Zhao et al. 2008).

However, the suggestion of the results in Figure 6.2 is that TRPC3 and TRPC6 are not involved in either the initial suggested inflammatory period, nor the later phase of mechanical hypersensitivity in these mice. The most likely explanation for this is a compensatory rebound in the population of other mechanically sensitive TRP channel populations, as already observed in SKO populations. These may not just be within TRPC, but extend across the whole family of previously characterised mechanosensitive TRP receptors, including TRPV4 and TRPA1(Suzuki, Mizuno et al. 2003, Kwan, Allchorne et al. 2006, Alessandri-Haber, Dina et al. 2009, Vilceanu and Stucky 2010). TRPA1 has similarly already been suggested as candidates for the generation of mechanical hypersensitivity in OA(McGaraughty, Chu et al. 2010, Chen, Joshi et al. 2011, Bautista, Pellegrino et al. 2013, Moilanen, Hämäläinen et al. 2015), though failure of other antagonist candidates make this picture less clear(*A.

OKUN1 2011). It is also possible that KO or antagonism of just one mechanosensitive TRP will always remain insufficient to provide meaningful analgesia due to the extent of redundancy within this system, and especially considering the associations and heteromeric ion channels that may form (Goel, Sinkins et al. 2002).

It is similarly possible that mechanosensory machinery other than the TRP channel underlie the development of mechanical hypersensitivity during OA. For example, increasingly evidence is pointing to proteins such as Piezo2, found through out subsets of myelinated and unmyelinated afferents (Delmas and Coste 2013). While the KO of this protein is deadly (Dubin, Schmidt et al. 2012), the use of antisense oligonucleotides have established the contribution of this protein to both inflammatory and neuropathic mechanical allodynia (Eijkelkamp, Linley et al. 2013).

6.4.4 Study Limitations

In considering the results and potential conclusions of this chapter, it is important to consider the limitations of the present study's design – both to provide context that will inform the conclusions, but also suggest potential modifications to the study that would have allowed greater clarity.

Evidence of Structural Pathology

A major limitation in the discussion of the results presented in this chapter, as in all the chapters of this thesis, is the absence of analysis of the structural pathology of these animals. Previous literature has established the histopathological changes associated with this doses (Horváth, Tékus et al. 2016), and lower doses (Yoon, Won et al. 2015, Uchimura, Foote et al. 2016), of MIA at the time points investigated in mice. As seen in the rat, these studies describe roughened cartilage surface, disorganization and cell loss, reduced matrix staining, and disrupted tidemark integrity with 0.5mg at day 22 (Horváth, Tékus et al. 2016). With a dose of 0.2mg one study reported observable necrotic clefts in the cartilage at 14 days (Yoon, Won et al. 2015), while at doses as low as 62.5µg OARSI scores of 4 were reported at day 10 (Uchimura, Foote et al. 2016).

However, it would be a fallacy to assume that every injection of MIA was successfully delivered. Even in a clinical setting, IA therapies commonly miss their mark (in much larger knees) without ultrasound guidance (Berkoff, Miller et al. 2012). There are similarly considerations of the potential off-target effects of MIA, such as leaking from the synovial joint to affect surrounding tissues and drive pain independently of OA-like pathology – including potential uptake by local neuronal endings to trigger neuropathic pain. These possibilities undermine the assumption that a pain profile necessarily demonstrates a successful injection and the presence of OA-like changes in the knee joint.

The conclusions drawn in this study are based upon potential considerations of the extent of histopathological changes and the development of an OA like condition of the knee – including the potential existence of an inflammatory phase in the earlier time points. These conclusions would be far stronger with a) evidence of such changes, on which to base these conclusions and b) a correlation analysis, to detect any potential relationship between the extent of structural pathology and behaviour. Such analysis could also allow the exclusion from analysis of animals in which OA had not been successfully induced by the MIA model. It is possible that in so doing, more significant differences between groups might be detected. Conversely, in a meta analysis of studies that measured behavioural pain outcomes in small animal models of OA, Suokas et al 2014 demonstrate that *“Lack of reported evidence that OA structural change was successfully induced in the model was strongly associated with larger effect sizes”*, where effect size refers to reported analgesic efficacy(Suokas, Sagar et al. 2014). This analysis suggests that incomplete phenotyping of animals, including the failure to confirm structural pathology, may lead to false conclusions. In this instance, the false conclusion would be to attribute differences in the response to the different genetic profiles of these mice, when these differences may instead related to off target effects of MIA in tissues surrounding the joint capsule.

Evidence of inflammation or neuropathy

The informed discussion of the likely mechanisms underlying the timeline of the changes in behavioural profile of MIA injected mice is limited by my failure to characterize directly any inflammation or signs of neuropathy in this model.

As mentioned at the beginning of 6.4.1, while we know much about the rat models of MIA induced OA, and extensive work has been published to build a confident picture of the histopathology, behaviour, inflammation and neuropathy at various doses, comparatively much less work has been performed in mice – with a degree of variability in the exact dose selected. It would be misleading to assume the initial inflammation seen in rats(Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Clements, Ball et al. 2009, Orita, Ishikawa et al. 2011, Ahmed, Li et al. 2012), or the development of neuropathy seen in rats at higher doses, would translate directly into a mouse MIA model(Ivanavicius, Ball et al. 2007, Orita, Ishikawa et al. 2011, Thakur, Rahman et al. 2012). While there is published work to suggest inflammation in a contributor to the mouse MIA model(van der Kraan, Vitters et al. 1989, Ogonna, Clark et al. 2012, Horváth, Tékus et al. 2016, Uchimura, Foote et al. 2016), relatively little exists to support the presence of neuropathy at this dose. Indeed, some studies have suggested this is unlikely in the 0.5mg model(Ogonna, Clark et al. 2012).

In order to validate the suggestions of this chapter future supplementary work would be required, including the measurement of joint diameter, knee histology at days 1, 3 and 7-post MIA injection,

profiling of ATF-3, microgliosis, and intra-epidermal nerve fiber density. Much as with joint histopathology above, this would allow a clearer picture of the timeline of pathology, how it corresponded with the hypersensitivity observed, and the differences observed between WT and DTA mice.

Failure to use sham, saline injected mouse controls

While the genetic profile of the mice was the key experimental variable under investigation in the above studies, with wild type littermates acting as controls, the failure to use a non-MIA, saline injected control cohort of mice can also be viewed as a major limitation to the conclusions of this study.

The original decision not to conduct a saline injected control stemmed from a desire to limit the number of experimental animals used. Conscientious animal research requires experimenters to find the balance between sufficiently powered experiments and the use of the minimum number of animals required, to avoid unnecessary wastage of animal lives and suffering. The decision also considered the challenges of breeding sufficient mice to allow appropriate power in four (instead of two) experimental arms in the DTA mouse experiments (DTA+MIA, DTA+Saline, WT+MIA, WT+Saline), and in the case of the TRP mouse lines up to 8 arms versus the four used.

While some published studies clearly draw the same conclusion, opting to not use a sham control arm in their pain studies in genetically manipulated mouse lines (Minett, Nassar et al. 2012, Horváth, Tékus et al. 2016), there are many documented MIA mouse studies which include a minimum of one saline sham control arm (So, Haraguchi et al. 2015), as also seen in MIA studies in non genetically manipulated mice (Harvey and Dickenson 2009, Ogbonna, Clark et al. 2012). These studies demonstrate the absence of effect from a saline injection to the knee over time, confirming that the injection of a similar volume of liquid using a 30G needle does not in and of itself, independent of the effect of MIA on chondrocytes, induce pain in the knee at any time-point studied (Harvey and Dickenson 2009, Ogbonna, Clark et al. 2012, So, Haraguchi et al. 2015). It would however have been valuable to establish, in a minimum of one arm (likely WT+Saline) that the injection procedure performed with myself as the experimenter was not the key driver of the change in behaviour observed within these mice.

6.4.5 Overall Implications

In the present chapter I have presented results that demonstrate that this 0.5mg MIA model induced both primary and secondary hypersensitivity in wild type, DTA, TRPC3 or TRPC6 SKO and DKO mouse lines, indicative of the development of central sensitization.

The absence of changes in punctate mechanical sensitivity during the early phase of this MIA model in the DTA mice could be viewed as further evidence of an initial inflammatory phase. It similarly suggests that the blockade of Nav1.8 may provide effective analgesia during this inflammatory flares or synovitis in patients with OA pain. It remains unclear what mechanisms may drive the behaviour observed in the later stages of this mouse model, however the similarities between the WT and DTA mice suggest these mechanisms are independent of Nav1.8 containing neurones.

Finally this work has suggested that the TRPC3 and TRPC6 receptors are not crucial contributors to the development of mechanical sensitivity in this model of OA pain, despite their role in innocuous mechanosensation. Though these channels may become sensitized to contribute to mechanical allodynia during OA, the scale of redundancy in the TRP mechanosensory system renders the KO of any one or two channels unlikely to effectively protect against the development of OA pain.

Chapter 7 – General Discussion

7.1 General Summary Of Findings

The experiments performed and presented herein aimed to further understand the differing contributions of peripheral and central mechanisms in the development and maintenance of pain in OA. Particular emphasis was placed on understanding how these contributions might differ with time and dose in a model of OA pain that has been demonstrated to involve dose and time dependent inflammatory, chronic nociceptive and neuropathic pain aetiologies (Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Ivanavicius, Ball et al. 2007, Thakur, Rahman et al. 2012).

This thesis has demonstrated that while a significant behavioural profile develops in both the early and late stages of the 1mg MIA model of OA pain, these changes are only reflected in L5 Lamina V WDR evoked responses during the early, inflammatory stages of the model. This suggests that the punctate hypersensitivity at the paw observed at day 3 and day 10 are driven by different spinal mechanisms, in the absence of L5 central sensitization at day 10.

I have similarly demonstrated that in a 1mg MIA model of OA pain, at both early and late stages, there is an absence of significant descending serotonergic facilitation and noradrenergic inhibition, in direct contrast to previous work in the 2mg model (Rahman, Bauer et al. 2009, Burnham and Dickenson 2013). Recordings in the brainstem, which failed to characterize significant adaptations in the activity of ON cells, corroborated these conclusions by suggesting that the 1mg MIA model failed to recruit descending controls.

The overarching suggestion of this work with the 1mg MIA model in rats is that OA pain lacking neuropathic components fails to recruit the descending control of spinal cord excitability. At days 10-14 it seems likely that joint pathology fails to drive ongoing nociception from the knee joint or inflict consequential damage to local sensory endings, and subsequently neither drives spreading central sensitization to the L5 segment serving the paw, nor recruit the intensity dependent descending controls. As such, I have suggested that differences in the aetiology of pain in the 1mg and 2mg models should directly impact the extrapolation of results to the clinic for development of pharmacotherapy in OA patients.

Finally, I have demonstrated a delay to the development of punctate mechanical sensitivity in DTA mice to day 7 in a 0.5mg MIA model of OA pain, potentially presenting further evidence of an initial inflammatory phase in this model. It similarly suggests that the blockade of Nav1.8 may provide effective analgesia during this inflammatory flares or synovitis in patients with OA pain. It remains unclear what mechanisms may drive the behaviour observed in the later stages of this mouse model.

7.2 Translational Relevance to the OA Clinic

The value of work conducted at the bench is in part defined by how it can inform our understanding of pain in a real world, clinical setting. The work presented within raises an important question about the relevance of conclusions drawn from pharmacology studies in the MIA model to a heterogeneous condition like OA pain. It similarly poses important questions around the OA patient segments that may best respond to certain interventions.

7.2.1 MIA as a Model of OA Pain – The Question of Neuropathy?

As part of this thesis, clear differences in the pharmacological profile of rats 10-14days after 1mg MIA versus previous work in 2mg MIA have been demonstrated. While the implied differences in the recruitment of descending controls could be attributed to the differing extent of joint pathology, questions have been raised about the possible contribution of neuropathy to the differences observed at the higher dose. The pertinent question here is whether neuropathy is driven by “on target” effect of MIA, namely joint degeneration damaging sensory endings in the joint, or an “off target” effect, such as possible leakage of MIA from the synovial joint to effect neighbouring endings outside the joint.

A simple proof of concept study is the injection of blue dye into the synovial joint of an adult rat cadaver. Working on the assumption of a similar diffusion pattern between the dye and saline solution containing MIA, the spread of this dye could be taken to model the reach of MIA after joint injection – though in a live animal this would be expected to extend further given both joint movement, body heat and bloody supply. It can be observed that the dye is not entirely confined to the synovial capsule, with spread into the neighbouring ligaments and muscle, all of which will be highly innervated. However it has previously been suggested that given fastblue is not taken up by neurones beyond the IA space following it's IA injection that perhaps MIA similarly fails to effect neurones beyond the IA space(Thakur 2012). Similarly Ivanavicius et al 2007 suggest that the time course of ATF-3, a marker of neuropathy, coincides with osteoclast activity which may drive the nerve damage, through a similar mechanism to that described in bone cancer pain(Ivanavicius, Ball et al. 2007). In reality, for each rat injected with MIA there may be small contributions of the former and small contributions from the latter to drive an overall pain profile. There is no definitive answer on the origins of the neuropathy.

While there is no clear-cut conclusion regarding the origins of neuropathy in the MIA model, it is questionable how important this is given the aetiology of neuropathy does not directly determine the pain profile of patients. Previous work characterizing the neuropathic pain profiles into distinct sub groups, based upon patters of gain and loss of function, have shown that while certain profiles may be

more common to certain diseases they are not exclusive(Baron, Tölle et al. 2009, Maier, Baron et al. 2010, Baron, Förster et al. 2012). As such the question is not what causes neuropathy in the 2mg model of MIA, but whether neuropathy itself is reflective of the clinical reality, and how differences in the pharmacology between 1mg and 2mg models may be similarly reflected in patient populations.

As discussed briefly in Section 3.1.5, while not reflective of the majority there is a sub population of OA patients with pain characteristics and sensory changes indicative of neuropathy(Hurley, Scott et al. 1997, Sharma 1999, Hawker, Stewart et al. 2008, Shakoor, Agrawal et al. 2008, Felson, Gross et al. 2009, Gwilym, Keltner et al. 2009, Shigemura, Ohtori et al. 2011, Hochman, Davis et al. 2013, Oteo - Álvaro, Ruiz - Ibán et al. 2014). The use of modified pain questionnaires and QST has suggested that ~20% of OA patients may have neuropathic contributions to their pain(Hochman, Davis et al. 2013), though some predictions are as high as 33%(Oteo - Álvaro, Ruiz - Ibán et al. 2014). As reviewed by Thakur et al 2014, nerve lesions, denervation and *de novo* innervation in OA patients have been characterized following samples during joint replacement surgery(Thakur, Dickenson et al. 2014). As such it can be argued that use of data from rats which have received doses of MIA ≥ 2 mg, at roughly day 14 onwards, may model pain reflective of this not unsubstantial group of neuropathic OA patients – this could be estimated to be as many as 1.7million people in the UK alone(Smith 2012). Meanwhile data from rats that have received doses < 2 mg may be a more representative model for the majority of patients, whose pain is driven by a mixture of inflammation and chronic nociception.

Thus, it could be postulated that the MIA model may provide a useful tool for investigating differences in the pain properties and pharmacological response profiles of patients with neuropathic or non-neuropathic characteristics. This could allow the optimization of pharmacotherapy for more successful analgesia in OA, such that prescriptions could become personalized to the individual patient profile. Examples of the relevance can be seen from the failure of gabapentinoids in OA Pain(Vedula, Bero et al. 2009). Previous work using the MIA model has shown that pregabalin is effective in the 2mg but not the 1mg model (Thakur, Rahman et al. 2012)– as such it may be the case that if patients had been segmented by the qualities of the pain, using QST or a modified PainDETECT questionnaire, the end points may have been met in the smaller neuropathic sub group. This concept has been demonstrated in a small trial of 89 patients using a combination of pregabalin and meloxicam, which demonstrated that patients with higher PainDETECT scores reported greater analgesia than those whose pain had fewer neuropathic qualities(Ohtori, Inoue et al. 2013).

At present the use of pain questionnaires, QST and enriched enrollment is incredibly rare in clinical trials of OA pain. At present, Clinicaltrials.gov lists just two OA trials testing pharmacological intervention that utilize PainDETECT or QST as an end point, and one of these was terminated prior to completion. Given the differences observed in pregabalin sensitivity depending on the dosage of MIA,

it is possible that we will continue to experience clinical failures in OA pain unless we segment patient groups.

7.2.2 Manipulating Descending Controls to Achieve Analgesia In OA Patients with Neuropathic Pain Qualities

The results presented within this thesis have suggested that while previous work has demonstrated that descending noradrenaline and serotonin may be recruited to the control of spinal excitability during a 2mg MIA model of OA(Rahman, Bauer et al. 2009, Burnham and Dickenson 2013), this is not the case during the 1mg model. The broad implication to the clinic is that interventions like duloxetine (Cymbalta), an SNRI used for both the management of mood and pain conditions, might provide more effective analgesia in those patients classified as having pain with neuropathic like qualities. Cymbalta is licensed in the US for musculoskeletal pain (including the management of OA) but failed to gain approval in the EU after “unfavourable risk-benefit balance” because “the clinical relevance of the effect is not established”((EMA) 2012). It is interesting to consider whether, had Lilly opted to segment patients using QST or questionnaires, whether they would have defined a sub group of patients in which efficacy was “clinically relevant” as per the reviewer’s requirements.

7.2.3 Viability of Nav1.8 for Analgesia in OA Pain

The translation of the implications of the MIA induced pain profile in DTA mice is a little less straightforward. The results of this study suggest that Nav1.8 antagonism might provide successful analgesia to patients in the earlier, inflammatory stage of OA pain, where pain is driven mostly by inflammation and mechanical nociception.

However, the ablation of a complete population of neurones based on the expression of Nav1.8 is not the equivalent of successful pharmacological blockade of Nav1.8. While antagonizing these channels should theoretically provide a degree of disruption of transmission in this population, the expression and action of Nav1.7 could be postulated to be sufficient to maintain the transmission of noxious mechanical sensation and inflammatory pain, based on work comparing the profiles of Nav1.8 KO and Nav1.7-Nav1.8 DKO mice(Nassar, Levato et al. 2005). While the neuropathic pain profile was indistinguishable across these two mouse populations and WT mice, only the DKO mouse exhibited increased noxious mechanical thresholds and resistance to inflammatory pain, while the Nav1.8 single KO mouse had a largely similar profile to the WT mice(Nassar, Levato et al. 2005). As such, there appears to be a critical role of the combined loss of Nav1.7 and Nav1.8 transmission to achieve

analgesia, which may suggest that an antagonist that shows selectivity for just Nav1.8 may fail where a dual antagonist succeeds.

Similarly, consideration must also be paid to the effect of chronic use of any pharmacological agent. Namely we must consider whether chronic antagonism of any sodium channel might result in the compensatory up-regulation of the target channel, to drive the development of tolerance such that larger doses are required, increasing the risk that effects are seen at off target channels in the heart, but also compensatory up-regulation or down-regulation of other channels and proteins. As has already been mentioned, the KO of Nav1.8 results in a compensatory increase in the expression of Nav1.7(Akopian, Souslova et al. 1999). In a chronic disease like OA, where dosing could conceivably continue for a matter of years with a well-tolerated drug, such modifications must also be considered. These adaptations are seen within opiate and monoamine systems(Pan 2007, Fava and Offidani 2011), where it would be interesting to characterize if this is the case for sodium channels.

Finally, consideration must also be given to those Nav1.8 antagonists already in development. In direct contrast to the conclusions I draw from KO animals, these studies claim analgesic efficacy of *selective* Nav1.8 antagonists in both inflammatory and *neuropathic* pain conditions in animal models, in direct contrast to expectations from genetic studies(Jarvis, Honore et al. 2007, Payne, Brown et al. 2015). However, the newest compound is yet to demonstrate efficacy in a clinical setting, where translation between animal models into patients have presented challenging – with recent failures in post-surgical dental pain(Skerratt and West 2015). It is possible that while blockade of Nav1.8 can provide some degree of relief that this analgesia will only achieve clinical relevance in combination with a Nav1.7 blocker – also in extensive clinical development at present.

7.3 Methodological Considerations

No experimental technique for the pre-clinical investigation of pain mechanisms is a perfect representation of clinical reality, however our awareness of the limitations give context to the conclusions we draw.

7.3.1 Further considerations when using the MIA model

Beyond considerations of the contributions of neuropathy to the pain profile of rats following the injection of different doses of MIA, it is important to understand in more depth the ultimate relevance of observations from the MIA model to the clinical reality of OA. As discussed briefly in Section 3, a large body of work suggests the MIA model effectively simulates the histopathology, hypersensitivity and disease progression observed in patients.

Common to both patients and rats are periods of inflammation(Fernandez-Madrid, Karvonen et al. 1994, Bove 2003, Guzman, Evans et al. 2003, Fernihough, Gentry et al. 2004, Bonnet and Walsh 2005, Clements, Ball et al. 2009); cartilage loss, fibrillation and compositional changes(Dunham, Hoedt-Schmidt et al. 1992, Poole 1993, Guingamp, Gegout-Pottie et al. 1997, Eyre 2004); and eventual bone remodeling, including osteophyte formation, cysts, and lost density(Kean, Kean et al. 2004, Wieland, Michaelis et al. 2005, Mohan, Perilli et al. 2011). However while the similarities between these structural changes are clear, the differences in the time course and aetiology must be considered. While the MIA model presents a time course with an established initial week of inflammation, synovitis in patients can be episodic – observed in both the early years and severe, late stages(Benito, Veale et al. 2005, D'Agostino, Conaghan et al. 2005). As such, testing during the second week and beyond in MIA animals may fail to characterize inflammatory components observed in the general OA population, which themselves may be an important contributor to pain. Periods of synovitis would similarly show different response properties to pharmacotherapy – perhaps this may explain how NSAID efficacy is lost during the late stages of the MIA model yet NSAID pharmacotherapy persists in the clinic(Fernihough, Gentry et al. 2004, Pomonis, Boulet et al. 2005).

Given a role for descending controls in inflammatory pain has previously been established(Green, Lyons et al. 1998, Green, Scarth et al. 2000, Rahman, Suzuki et al. 2004), the impact of this on this on the conclusions I have drawn, that pharmacotherapies manipulating descending controls such as duloxetine may not be effective in non neuropathic OA pain, may be too simplistic. It may be that SSRIs and SNRIs may be effective in patients with either inflammatory flares or neuropathic pain characteristics, but not those patients in a period where pain is driven by neither of the above.

There similarly remain questions about the presence or not of central sensitization during the MIA model. As was highlighted in the discussions of Section 3, there is no clear-cut consensus on whether the injection of MIA enhances the evoked responses of WDR cells in lamina V. While 3 studies point to significant increases in the responses evoked by vF(Harvey and Dickenson 2009, Rahman, Bauer et al. 2009, Burnham 2012), another 3 failed to replicate this(Vonsy 2008, Patel 2012, Thakur 2012). In part, this may be attributed to the sample size, where cell populations of just $\sim n=7$ per group may allow one or two very active cells to skew the data. However, in the case of both Burnham et al 2012 and Thakur et al 2012, data for a number of studies were compiled to provide study populations of between 30-45 cells(Burnham 2012, Thakur 2012), which statistically would be expected to even this effect out. It would be revealing to pool the data across the 5 rat studies cited, along with other historical lab data to create a stronger consensus. As it stands, my data suggests there is not – at least in the 1mg MIA model – a clear spreading central sensitization in these animals in the late stages of the model. This creates questions about the validity of a 1mg model for investigating OA pain given the clear consensus around the presence of central sensitization in the clinic. This may again link back to the question of inflammation, since I did demonstrate central sensitization during the early inflammatory phase. Perhaps in the clinic, central sensitization is maintained by evolving contributions of inflammation, mechanical nociception and neuropathy - whereas in the 10-14day period of the 1mg MIA model there is not sufficient drive to maintain central sensitization from either of these mechanisms. It is possible instead that this time point in the 1mg MIA model may better represent the very early stages of OA, before the patient seeks medical assistance or progresses beyond paracetamol for the management of their pain.

7.3.2 Limitations of *in vivo* Electrophysiology

in vivo electrophysiology is an exceptionally valuable tool, providing quantitative information of the responses of neurones in the PNS and CNS to suprathreshold stimuli, both in healthy animals and pathological conditions. The value of these techniques above behaviour, though the information is complimentary, is allowing experimenters to develop an understanding of changes that would otherwise be unobservable from behaviour, or even unethical to pursue – namely hyperalgesia. For example, that a drug may quench the responses of a lamina V WDR to 50°C water may be observed using spinal electrophysiology, however if the evoked response is still greater than the withdrawal threshold this may not be observed with behaviour.

However, there are limitations to the technique, which are applicable for both the recordings made from the spinal cord and the RVM. The process of cell searching, which crudely involves the application of a stimulus while listening and looking for responses on an oscilloscope while gently altering the electrodes position, is heavily biased by experimenter selection and “low hanging fruit” cells, which may skew cell sample demographics. The first is demonstrated by the differences in baseline responses observed in naïve animals between different experimenters – take Thakur et al 2012, which characterized a mean response to 60g vF >1100 action potentials, while Burnham et al characterized a mean nearer 800 action potentials(Burnham 2012, Thakur 2012), despite study populations of n=21 and n=39 respectively. Such differences are understood to be driven by the experimenter selection process, where one individual may be more likely to characterize more “noisy” and active cells than another, for a variety of reasons. In a similar vane, the concept of “low hanging fruit” cells – cells which are more active, with larger evoked responses or a degree of spontaneous activity will be easier to identify and perhaps more attractive to follow, to the disadvantage of cells with a more phasic or sedate response profile. As such, the conclusions drawn from any study may only apply to one population of many within both the spinal cord and the brain.

This limitation especially holds true for characterization of cells in the RVM, where the search for specific response profiles may cause the experimenter to fail to identify cells or move on because during that particular time period the cell was silent or unresponsive. This is especially relevant to the study of chronic pain conditions, since there would be huge value in knowing if perhaps chronic pain silences or causes identity switched in cells of the RVM. While population studies may seem like a viable alternate, they may be misleading given these too are inherently biased by the search strategy and operator choice.

7.3.3 Limitations of KO Mice for the Study of Pain Mechanisms

The use of knock out mice for the study of healthy and pathological pain has been a valuable tool for experimenters, where the deletion of genes may allow direct inferences about function based on the resulting phenotype. However, the tool is not perfect – the deletion of one gene can commonly have no effect at all, unexpected effects in out of scope systems or simply result in death of the mouse (Barbaric, Miller et al. 2007)– as was the case for Nav1.7 global KO mice(Minett, Nassar et al. 2012). Relevant to this work is the lack of effect on mouse phenotype, where failing to characterize a difference following double KO of TRPC3 and TRPC6 does not necessarily exclude these proteins from the generation of allodynia during OA. Another functional mechnoceptor “steps in” to make up the difference, as is seen in SKO mice and blood pressure(Spassova, Hewavitharana et al. 2006). This

functional redundancy may hide the involvement of TRPC3 and TRPC6 in the development of mechanical allodynia in the MIA model of OA pain – though regardless it may suggest this is a poor pharmacological target for the treatment of allodynia.

7.4 Further Research

A continuing suggestion and question following these studies is whether the failure to demonstrate central sensitization and recruitment of descending controls from the brainstem at days 10-16 is a time sensitive conclusion. In previous studies in which 28+ day end points are used, after doses of both 1mg or 2mg MIA, this model of OA is demonstrated to be more pronounced. Sagar et al 2010 similarly failed to demonstrate any alterations in spinal cord excitability at days 14-17 to punctate stimulation, but characterizing a significant sensitivity at days 28-31 to mid-range punctate stimuli that correlated strongly to joint pathology(Sagar, Staniaszek et al. 2010). A similar time dependent recruitment of descending control has been observed from the “unmasking” effect of spinalizations at the 28th day of a 1mg MIA model(Kelly, Dobson et al. 2013), and the time dependent efficacy of a NA re-uptake inhibition to resolve incapacitance at week 3 of the 1mg model(Whiteside, Dwyer et al. 2010). As such, it would be interesting to characterize if later days in the 1mg model, perhaps at days 28 and 35, enhancements of evoked responses of WDR neurones and recruitment of descending serotonergic and noradrenergic controls can be observed. This would further support conclusions made within that at days 10-14 of the 1mg model that the drive from the arthritic knees is not yet sufficient to recruit descending controls, as the 2mg MIA model does. It would be similarly interesting to characterize the RVM at these later days of the 1mg MIA model, and extend RVM studies into the 2mg MIA model to demonstrate any differences in the activity of RVM neurones in both a more advanced joint pathology and a mildly neuropathic OA pain model.

These studies have similarly only looked to characterize descending serotonergic facilitation in the 1mg MIA model. Previous work has demonstrated that at the late stages of the 2mg MIA model descending 5HT acting at the 5HT₇ receptor provided a tonic regulation of thermal processing that was increased versus naïve animals, likely as a consequence of differing joint pathology(Burnham 2012). Conversely, the tonic inhibition of mechanically evoked responses through 5HT at this receptor was much smaller in the MIA animals than in naïve animals. This suggests that in addition to adaptations in facilitatory serotonergic systems(Rahman, Bauer et al. 2009), similar adaptations may occur in the serotonergic inhibitory system. In parallel the above suggested studies, there would be value in completing the picture by gaining an understanding of the contribution of 5HT acting at 5HT₇ at days 3, 14 and 28 of the 1mg MIA model.

Finally there may be value in exploring these changes to descending control systems in a model of OA pain that does not involve the introduction of a toxin into the knee, but instead follows an aetiology more similar to OA in the clinic with the more ongoing presence of inflammation discussed above, such as the meniscal transection model. This model maintains a greater inflammation score across time points(Mapp, Sagar et al. 2013), unlike MIA, where the study of descending controls and Nav1.8

blockers following meniscal transection may provide greater insight to the monoaminergic contributions to pain in those patients experiencing synovitis/inflammatory flares.

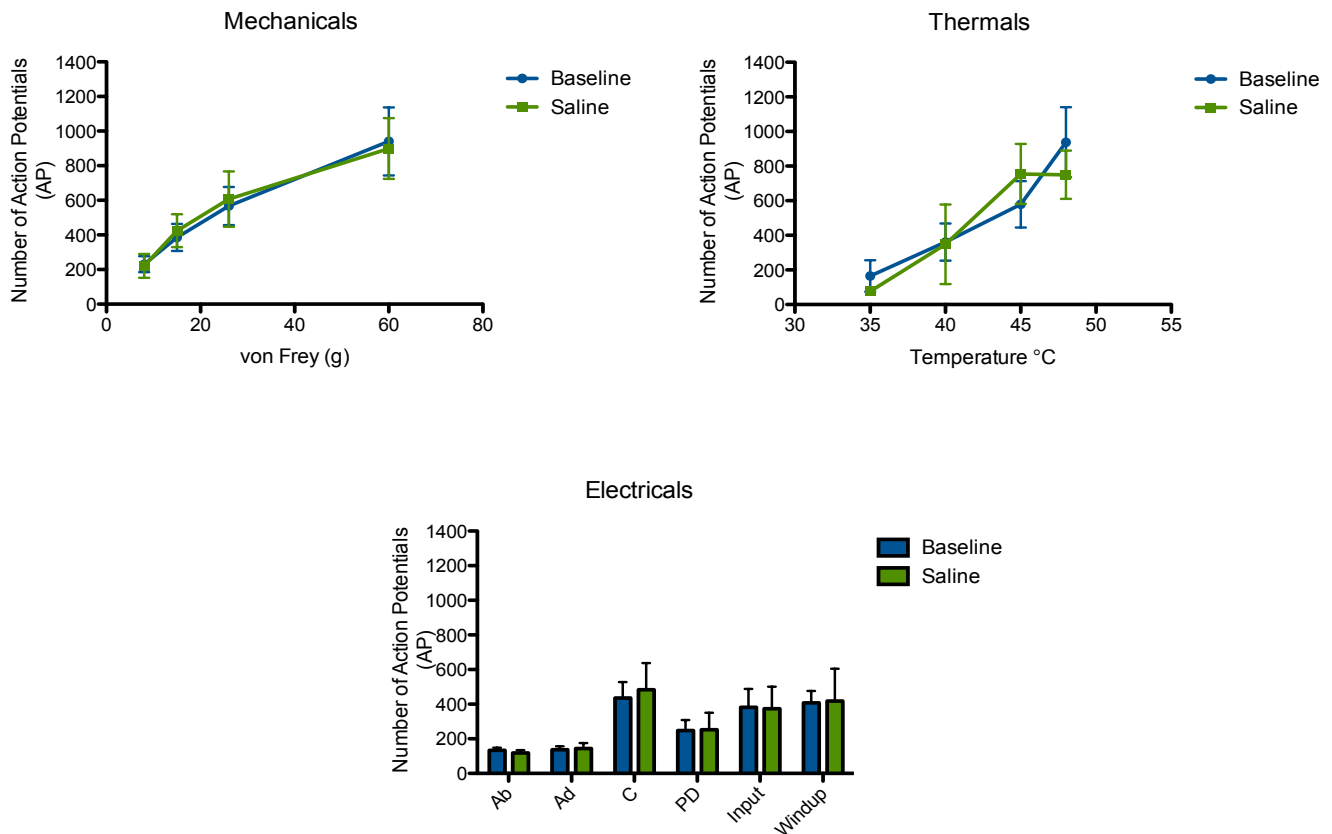
7.5 Closing Remarks

The work I have presented in this thesis, when considered in the context of previous work from this lab and others in the MIA model of OA pain, suggests that pain mechanisms, notably descending control of pain, will vary considerably between the different stages of OA, where the extent of joint pathology, inflammatory flares or neuropathy, themselves a factor of time from induction, will determine to what extent descending controls are recruited. The impact of these conclusions is the suggestion that successful analgesia in OA may rely on patient segmentation – sorting patients using QST and pain questionnaires and prescribing based on the pain profile, where the conclusions within suggest that those patients with the most neuropathic like qualities to their pain may benefit most from SNRI therapy, while those patients with inflammatory flares might benefit more from Nav1.8 blockade.

It is my hope that the studies presented within this thesis have contributed further to our understanding of the contributions to pain in OA, particularly with reference to the utility of manipulating descending controls to provide analgesia, and will help shape future approaches to pain management in OA pain.

Chapter 8 – Appendix

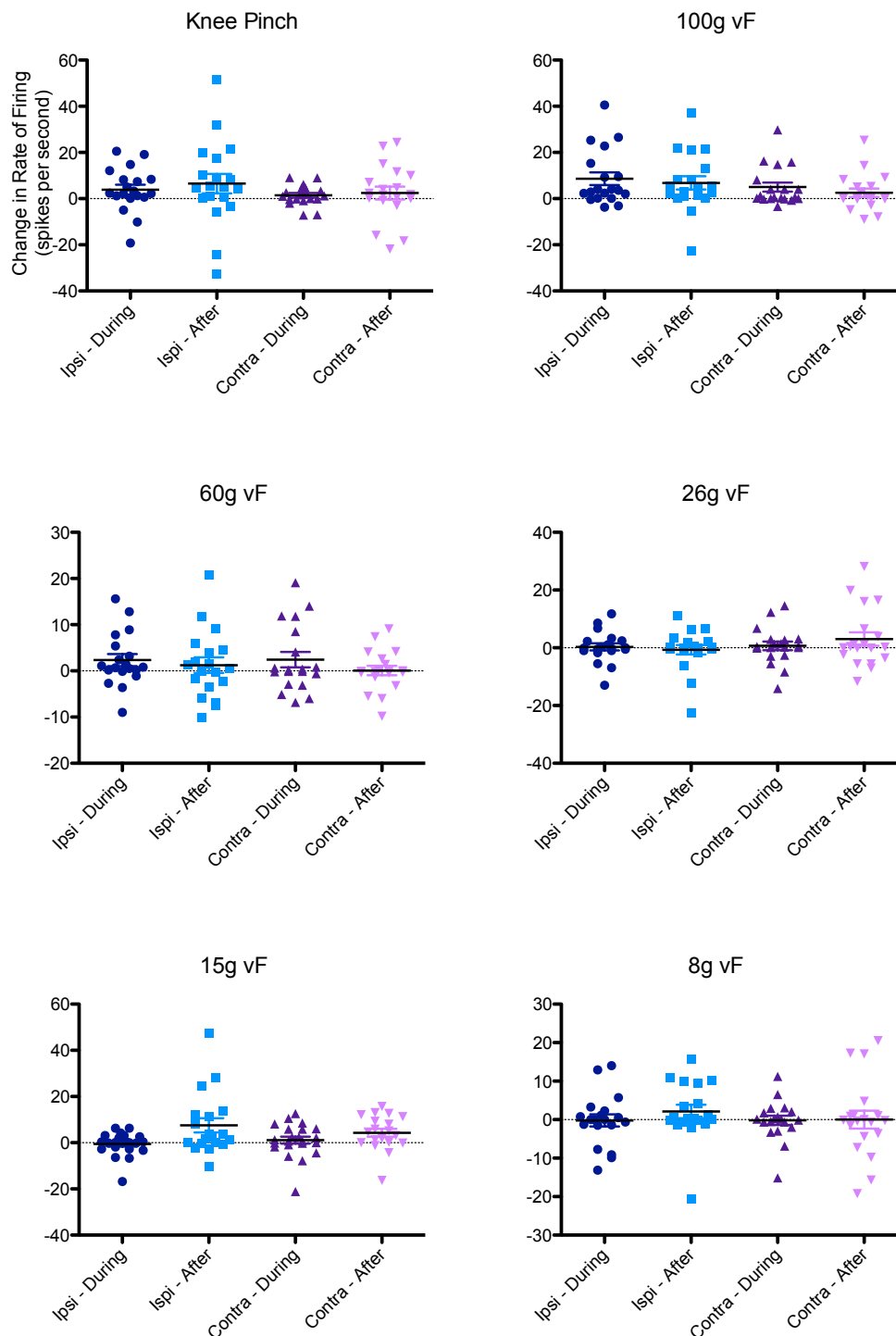
Appendix 1: Spinal Cord Electrophysiology – Saline Controls in Naïve Rats



Appendix 1 – Mechanical, thermal and electrically evoked responses of Lamina V WDR cells in naïve rats are unchanged following the spinal cord application of saline (n=5)

The evoked responses of Lamina V WDR cells following the application of vF hairs, thermal water jet or electrical stimulation to the paw in naïve rats is unchanged following the application of saline to the spinal cord. Evoked responses exhibit a stimulus intensity dependent increase in the number of action potentials with increasingly noxious mechanical or thermal stimulation. This stimulus dependent relationship is unchanged following saline.

Appendix 2: Comparison of ON Cell Responses To Somatic Simulation in MIA rats – During and After Stimulus



Appendix 2 - The response of RVM ON-cells to mechanical stimulation of the knee and paw in rats 14-16days after the IA injection of 1mg MIA – Maximum rate of change of firing of pre-characterized ON cells either during the 20second application or for the 20seconds after the removal of a mechanical stimuli – knee pinch, 100g, 60g, 26g, 15g or 8g von Frey. The greatest change in the rate of firing was observed in the “after” responses, which exhibit a greater SD in 10 out of 12 comparisons.

Chapter 9 - References

- (EMA), E. M. A. (2012). "CHMP Assessment Report - Cymbalta." from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Assessment_Report_-_Variation/human/000572/WC500128545.pdf.
- *A. OKUN1, P. L., P. DAVIS1, J. REN1, M. OSSIPOV1, Y. XIE1, G. DUSSOR1, T. KING1, F. PORRECA (2011). "Afferent drive produces ongoing pain in the MIA model of Osteoarthritis." 29-32.
- Abaei, M., D. R. Sagar, E. G. Stockley, C. H. Spicer, M. Prior, V. Chapman and D. P. Auer (2016). "Neural correlates of hyperalgesia in the monosodium iodoacetate model of osteoarthritis pain." Molecular pain **12**: 1744806916642445.
- Abrahamsen, B., J. Zhao, C. O. Asante, C. M. Cendan, S. Marsh, J. P. Martinez-Barbera, M. A. Nassar, A. H. Dickenson and J. N. Wood (2008). "The cell and molecular basis of mechanical, cold, and inflammatory pain." Science Signalling **321**(5889): 702.
- Abramson, S. B. (2004). "Inflammation in osteoarthritis." Journal of Rheumatology-Supplements **31**(70): 70-76.
- Ahmadi, S., S. Lippross, W. L. Neuhuber and H. U. Zeilhofer (2002). "PGE2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons." Nature Neuroscience **5**(1): 34-40.
- Ahmed, A. S., J. Li, H. Erlandsson-Harris, A. Stark, G. Bakalkin and M. Ahmed (2012). "Suppression of pain and joint destruction by inhibition of the proteasome system in experimental osteoarthritis." Pain **153**(1): 18-26.
- Aimone, L. and G. Gebhart (1986). "Stimulation-produced spinal inhibition from the midbrain in the rat is mediated by an excitatory amino acid neurotransmitter in the medial medulla." The Journal of neuroscience **6**(6): 1803-1813.
- Akopian, A. N., V. Souslova, S. England, K. Okuse, N. Ogata, J. Ure, A. Smith, B. J. Kerr, S. B. McMahon and S. Boyce (1999). "The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways." Nature neuroscience **2**(6): 541-548.
- Al - Khater, K. M. and A. J. Todd (2009). "Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area." Journal of Comparative Neurology **515**(6): 629-646.
- Alessandri-Haber, N., O. A. Dina, X. Chen and J. D. Levine (2009). "TRPC1 and TRPC6 channels cooperate with TRPV4 to mediate mechanical hyperalgesia and nociceptor sensitization." The Journal of neuroscience **29**(19): 6217-6228.
- Aloe, L., M. A. Taveri, U. Carcassi and R. Levi - Montalcini (1992). "Nerve growth factor in the synovial fluid of patients with chronic arthritis." Arthritis & Rheumatism **35**(3): 351-355.
- Altman, R. D., A. Aven, C. E. Holmburg, L. M. Pfeifer, M. Sack and G. T. Young (1994). Capsaicin cream 0.025% as monotherapy for osteoarthritis: a double-blind study. Seminars in arthritis and rheumatism, Elsevier.
- Andersen, O. K., R. Gracely and L. ARENDT - NIELSEN (1995). "Facilitation of the human nociceptive reflex by stimulation of A β - fibres in a secondary hyperalgesic area sustained by nociceptive input from the primary hyperalgesic area." Acta physiologica scandinavica **155**(1): 87-97.

Anderson, D. D., S. Chubinskaya, F. Guilak, J. A. Martin, T. R. Oegema, S. A. Olson and J. A. Buckwalter (2011). "Post - traumatic osteoarthritis: Improved understanding and opportunities for early intervention." Journal of Orthopaedic Research **29**(6): 802-809.

Anderson, L. E. and V. S. Seybold (2000). "Phosphorylated cAMP response element binding protein increases in neurokinin-1 receptor-immunoreactive neurons in rat spinal cord in response to formalin-induced nociception." Neuroscience Letters **283**(1): 29-32.

Andrew, D., K. E. Krout and A. Craig (2003). "Differentiation of lamina I spinomedullary and spinothalamic neurons in the cat." Journal of Comparative Neurology **458**(3): 257-271.

Arden, N. and M. C. Nevitt (2006). "Osteoarthritis: epidemiology." Best Practice & Research Clinical Rheumatology **20**(1): 3-25.

Arendt-Nielsen, L., H. Nie, M. B. Laursen, B. S. Laursen, P. Madeleine, O. H. Simonsen and T. Graven-Nielsen (2010). "Sensitization in patients with painful knee osteoarthritis." Pain **149**(3): 573-581.

Arendt-Nielsen, L., H. Nie, M. B. Laursen, B. S. Laursen, P. Madeleine, O. H. Simonsen and T. Graven-Nielsen (2010). "Sensitization in patients with painful knee osteoarthritis." Pain **149**: 573-581.

Arthritis Research, U. (Nov 2008). "Arthritis in the UK – key facts." Journal of Rheumatology: 5-8.

Avouac, J., L. Gossec and M. Dougados (2007). "Efficacy and safety of opioids for osteoarthritis: a meta-analysis of randomized controlled trials." Osteoarthritis and Cartilage **15**(8): 957-965.

Baba, H., T. P. Doubell and C. J. Woolf (1999). "Peripheral inflammation facilitates A β fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord." The Journal of Neuroscience **19**(2): 859-867.

Baba, H., T. Kohno, K. A. Moore and C. J. Woolf (2001). "Direct activation of rat spinal dorsal horn neurons by prostaglandin E₂." The Journal of neuroscience **21**(5): 1750.

Bajaj, P., T. Graven-Nielsen and L. Arendt-Nielsen (2001). "Osteoarthritis and its association with muscle hyperalgesia: an experimental controlled study." Pain **93**: 107-114.

Bajic, D. and H. K. Proudfit (1999). "Projections of neurons in the periaqueductal gray to pontine and medullary catecholamine cell groups involved in the modulation of nociception." The Journal of comparative neurology **405**(3): 359-379.

Balanescu, A. R., E. Feist, G. Wolfram, I. Davignon, M. D. Smith, M. T. Brown and C. R. West (2014). "Efficacy and safety of tanezumab added on to diclofenac sustained release in patients with knee or hip osteoarthritis: a double-blind, placebo-controlled, parallel-group, multicentre phase III randomised clinical trial." Annals of the rheumatic diseases **73**(9): 1665-1672.

Balkowiec, A. and D. M. Katz (2000). "Activity-Dependent Release of Endogenous Brain-Derived Neurotrophic Factor from Primary Sensory Neurons Detected by ELISA In Situ." The Journal of neuroscience **20**(19): 7417-7423.

Bandler, R. and M. T. Shipley (1994). "Columnar organization in the midbrain periaqueductal gray: modules for emotional expression?" Trends in neurosciences **17**(9): 379-389.

Bannister, K., R. Patel, L. Goncalves, L. Townson and A. Dickenson (2015). "DNIC and nerve injury: restoring an imbalance between descending monoamine inhibitions and facilitations." Pain.

Baraf, H. S. B., F. M. Gloth, H. R. Barthel, M. S. Gold and R. D. Altman (2011). "Safety and efficacy of topical diclofenac sodium gel for knee osteoarthritis in elderly and younger patients: pooled data from three randomized, double-blind, parallel-group, placebo-controlled, multicentre trials." Drugs & Aging **28**(1): 27-40.

Baragi, V. M., G. Becher, A. M. Bendele, R. Biesinger, H. Bluhm, J. Boer, H. Deng, R. Dodd, M. Essers and T. Feuerstein (2009). "A new class of potent matrix metalloproteinase 13 inhibitors for potential treatment of osteoarthritis: evidence of histologic and clinical efficacy without musculoskeletal toxicity in rat models." Arthritis & Rheumatism **60**(7): 2008-2018.

Barbaric, I., G. Miller and T. N. Dear (2007). "Appearances can be deceiving: phenotypes of knockout mice." Briefings in functional genomics & proteomics **6**(2): 91-103.

Barbaro, N. M., M. M. Heinricher and H. L. Fields (1986). "Putative pain modulating neurons in the rostral ventral medulla: reflex-related activity predicts effects of morphine." Brain research **366**(1): 203-210.

Barbaro, N. M., M. M. Heinricher and H. L. Fields (1989). "Putative nociceptive modulatory neurons in the rostral ventromedial medulla of the rat display highly correlated firing patterns." Somatosensory & motor research **6**(4): 413-425.

Bardin, L. (2011). "The complex role of serotonin and 5-HT receptors in chronic pain." Behavioural pharmacology **22**(5 and 6): 390-404.

Baron, R., M. Förster and A. Binder (2012). "Subgrouping of patients with neuropathic pain according to pain-related sensory abnormalities: a first step to a stratified treatment approach." The Lancet Neurology **11**(11): 999-1005.

Baron, R., T. R. Tölle, U. Gockel, M. Brosz and R. Freynhagen (2009). "A cross-sectional cohort survey in 2100 patients with painful diabetic neuropathy and postherpetic neuralgia: differences in demographic data and sensory symptoms." Pain **146**(1): 34-40.

Barthel, C., N. Yermenko, R. Jacobs, R. E. Schmidt, M. Bernateck, H. Zeidler, P.-P. Tak, D. Baeten and M. Rihl (2009). "Nerve growth factor and receptor expression in rheumatoid arthritis and spondyloarthritis." Arthritis Research and Therapy **11**(3): R82.

Basbaum, A. I., D. D. Ralston and H. J. Ralston (1986). "Bulbospinal projections in the primate: a light and electron microscopic study of a pain modulating system." Journal of Comparative Neurology **250**(3): 311-323.

Bautista, D. M., M. Pellegrino and M. Tsunozaki (2013). "TRPA1: a gatekeeper for inflammation." Annual Review of Physiology **75**: 181-200.

Bautista, D. M., J. Siemens, J. M. Glazer, P. R. Tsuruda, A. I. Basbaum, C. L. Stucky, S.-E. Jordt and D. Julius (2007). "The menthol receptor TRPM8 is the principal detector of environmental cold." Nature **448**(7150): 204-208.

Bee, L. a. and a. H. Dickenson (2007). "Rostral ventromedial medulla control of spinal sensory processing in normal and pathophysiological states." Neuroscience **147**: 786-793.

Bee, L. A. and A. H. Dickenson (2008). "Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin." Pain **140**(1): 209-223.

- Beitz, A. (1982). "The nuclei of origin of brain stem enkephalin and substance P projections to the rodent nucleus raphe magnus." *Neuroscience* **7**(11): 2753-2768.
- Beitz, A. J. (1990). "Relationship of glutamate and aspartate to the periaqueductal gray-raphe magnus projection: analysis using immunocytochemistry and microdialysis." *Journal of Histochemistry & Cytochemistry* **38**(12): 1755.
- Bellamy, N. (2008). *WOMAC osteoarthritis index: user guide IX*, Nicholas Bellamy.
- Bellamy, N., J. Campbell, V. Welch, T. Gee, R. Bourne and G. Wells (2009). "Intraarticular corticosteroid for treatment of osteoarthritis of the knee (Review)."
- Benito, M. J., D. J. Veale, O. FitzGerald, W. B. van den Berg and B. Bresnihan (2005). "Synovial tissue inflammation in early and late osteoarthritis." *Annals of the rheumatic diseases* **64**(9): 1263-1267.
- Berenbaum, F., F. Eymard and X. Houard (2013). "Osteoarthritis, inflammation and obesity." *Current opinion in rheumatology* **25**(1): 114-118.
- Berkoff, D. J., L. E. Miller and J. E. Block (2012). "Clinical utility of ultrasound guidance for intra-articular knee injections: a review." *Clin Interv Aging* **7**: 89-95.
- Berman, S. M., B. D. Naliboff, B. Suyenobu, J. S. Labus, J. Stains, G. Ohning, L. Kilpatrick, J. A. Bueller, K. Ruby and J. Jarcho (2008). "Reduced brainstem inhibition during anticipated pelvic visceral pain correlates with enhanced brain response to the visceral stimulus in women with irritable bowel syndrome." *The Journal of Neuroscience* **28**(2): 349-359.
- Beswick, A. D., V. Wylde, R. Gooberman-Hill, A. Blom and P. Dieppe (2012). "What proportion of patients report long-term pain after total hip or knee replacement for osteoarthritis? A systematic review of prospective studies in unselected patients." *BMJ open* **2**(1).
- Bijlsma, J. W., F. Berenbaum and F. P. Lefeber (2011). "Osteoarthritis: an update with relevance for clinical practice." *The Lancet* **377**(9783): 2115-2126.
- Bingel, U. and I. Tracey (2008). "Imaging CNS modulation of pain in humans." *Physiology* **23**(6): 371-380.
- Birder, L. and E. Perl (1999). "Expression of $\alpha 2$ - adrenergic receptors in rat primary afferent neurones after peripheral nerve injury or inflammation." *The Journal of physiology* **515**(2): 533-542.
- Birrell, F. H., Nick; Porchenret, Mark; (2011). "Topical Reviews and prospects for treatment." *Arthritis Research UK - Topical Reviews Series* **6**(10).
- Blagojevic, M., C. Jinks, A. Jeffery and K. Jordan (2010). "Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis." *Osteoarthritis and cartilage* **18**(1): 24-33.
- Bley, K. R., A. Bhattacharya, D. V. Daniels, J. Gever, A. Jahangir, C. O'Yang, S. Smith, D. Srinivasan, A. P. Ford and M. F. Jett (2006). "R01138452 and R03244794: characterization of structurally distinct, potent and selective IP (prostacyclin) receptor antagonists." *British journal of pharmacology* **147**(3): 335-345.
- Boegel, K., F. E. Gyulai, K. Moore and M. S. Gold (2011). "DELETERIOUS IMPACT OF γ -AMINOBUTYRIC ACID TYPE A RECEPTOR PREFERRING GENERAL ANESTHETIC WHEN USED IN THE PRESENCE OF PERSISTENT INFLAMMATION." *Anesthesiology* **115**(4): 782.
- Boettger, M. K., K. Weber, M. Gajda, R. Bräuer and H.-G. Schaible (2010). "Spinally applied ketamine or morphine attenuate peripheral inflammation and

hyperalgesia in acute and chronic phases of experimental arthritis." Brain, behavior, and immunity **24**(3): 474-485.

Bonnet, C. and D. Walsh (2005). "Osteoarthritis, angiogenesis and inflammation." Rheumatology **44**(1): 7-16.

Bonnington, J. K. and P. A. McNaughton (2003). "Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor." The Journal of physiology **551**(2): 433-446.

Bouhassira, D., Z. Bing and D. Le Bars (1993). "Studies of brain structures involved in diffuse noxious inhibitory controls in the rat: the rostral ventromedial medulla." The Journal of physiology **463**: 667.

Bove, S. (2003). "Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis." Osteoarthritis and Cartilage **11**: 821-830.

Bowles, R. D., B. A. Mata, R. D. Bell, T. K. Mwangi, J. L. Huebner, V. B. Kraus and L. A. Setton (2014). "In Vivo Luminescence Imaging of NF - κ B Activity and Serum Cytokine Levels Predict Pain Sensitivities in a Rodent Model of Osteoarthritis." Arthritis & Rheumatology **66**(3): 637-646.

Bowsher, D. (1997). "The effects of pre-emptive treatment of postherpetic neuralgia with amitriptyline: A randomized, double-blind, placebo-controlled trial." Journal of pain and symptom management **13**(6): 327-331.

Bradley, L. A., B. C. Kersh, J. J. De Berry, G. Deutsch, G. A. Alarcon and D. McLain (2004). Lessons from fibromyalgia: abnormal pain sensitivity in knee osteoarthritis. Novartis Foundation Symposium, Chichester; New York; John Wiley; 1999.

Brain, S., T. Williams, J. Tippins, H. Morris and I. MacIntyre (1985). "Calcitonin gene-related peptide is a potent vasodilator."

Bráz, J. M., L. Ackerman and A. I. Basbaum (2011). "Sciatic nerve transection triggers release and intercellular transfer of a genetically expressed macromolecular tracer in dorsal root ganglia." Journal of Comparative Neurology **519**(13): 2648-2657.

Breivik, H., B. Collett, V. Ventafridda, R. Cohen and D. Gallacher (2006). "Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment." European Journal of Pain **10**(4): 287-333.

Brenner, G. J., R. R. Ji, S. Shaffer and C. J. Woolf (2004). "Peripheral noxious stimulation induces phosphorylation of the NMDA receptor NR1 subunit at the PKC, Δ 896, in spinal cord dorsal horn neurons." European Journal of Neuroscience **20**(2): 375-384.

Brink, T. S. and P. Mason (2003). "Raphe magnus neurons respond to noxious colorectal distension." Journal of neurophysiology **89**(5): 2506-2515.

Brown, M. T., F. T. Murphy, D. M. Radin, I. Davignon, M. D. Smith and C. R. West (2012). "Tanezumab reduces osteoarthritic knee pain: results of a randomized, double-blind, placebo-controlled phase III trial." The Journal of Pain **13**(8): 790-798.

Brumovsky, P., M. Watanabe and T. Hökfelt (2007). "Expression of the vesicular glutamate transporters-1 and-2 in adult mouse dorsal root ganglia and spinal cord and their regulation by nerve injury." Neuroscience **147**(2): 469-490.

Buchanan, W. W. and W. F. Kean (2002). "Osteoarthritis III: Radiological and clinical definition." InflammoPharmacology **10**(1-2): 53-78.

Budai, D. and A. A. Larson (1996). "Role of substance P in the modulation of C-fiber-evoked responses of spinal dorsal horn neurons." Brain Research **710**(1-2): 197-203.

Budai, D., G. L. Wilcox and A. A. Larson (1995). "Effects of nitric oxide availability on responses of spinal wide dynamic range neurons to excitatory amino acids." European Journal of Pharmacology **278**(1): 39-47.

Burgess, S. E., L. R. Gardell, M. H. Ossipov, T. P. Malan, T. W. Vanderah, J. Lai and F. Porreca (2002). "Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain." The Journal of neuroscience : the official journal of the Society for Neuroscience **22**: 5129-5136.

Burnham, L. (2012). Monoaminergic control of the central processing of sensory stimuli in a rat model of osteoarthritis, University College London.

Burnham, L. J. and A. H. Dickenson (2013). "The antinociceptive effect of milnacipran in the monosodium iodoacetate model of osteoarthritis pain and its relation to changes in descending inhibition." Journal of Pharmacology and Experimental Therapeutics **344**(3): 696-707.

Burnstein, R., R. Dado and G. Giesler (1990). "The cells of origin of the spinothalamic tract of the rat: a quantitative re-examination." Brain Res. **511**: 329-337.

Burnstock, G. (1996). "A unifying purinergic hypothesis for the initiation of pain." The Lancet **347**(9015): 1604-1605.

Burston, J., L. Xu, U. Grabowska, K. Tunblad, E. Lindström and V. Chapman (2016). "The Cathepsin K inhibitor L-006235 has analgesic and disease modifying properties in the MIA model of osteoarthritis." Osteoarthritis and Cartilage **24**: S454.

Burston, J. J., D. R. Sagar, P. Shao, M. Bai, E. King, L. Brailsford, J. M. Turner, G. J. Hathway, A. J. Bennett and D. A. Walsh (2013). "Cannabinoid CB 2 Receptors Regulate Central Sensitization and Pain Responses Associated with Osteoarthritis of the Knee Joint." PloS one **8**(11): e80440.

Calza, L., M. Pozza, M. Zanni, C. Manzini, E. Manzini and T. Hökfelt (1997). "Peptide plasticity in primary sensory neurons and spinal cord during adjuvant-induced arthritis in the rat: an immunocytochemical and *in situ* hybridization study." Neuroscience **82**(2): 575-589.

Cameron, M., F. Fu, H. Paessler, M. Schneider and C. Evans (1994). "Synovial fluid cytokine concentrations as possible prognostic indicators in the ACL-deficient knee." Knee Surgery, Sports Traumatology, Arthroscopy **2**(1): 38-44.

Cardenas, C. G., L. P. Del Mar and R. S. Scroggs (1997). "Two parallel signaling pathways couple 5HT_{1A} receptors to N- and L-type calcium channels in C-like rat dorsal root ganglion cells." Journal of neurophysiology **77**(6): 3284-3296.

Carlson, J. D., J. J. Maire, M. E. Martenson and M. M. Heinricher (2007). "Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury." The Journal of Neuroscience **27**(48): 13222-13231.

Carr, F. B., S. M. Géranton and S. P. Hunt (2014). "Descending controls modulate inflammatory joint pain and regulate CXCL chemokine and iNOS expression in the dorsal horn." Molecular pain **10**(1): 39.

Carstens, E. and D. L. Trevino (1978). "Laminar origins of spinothalamic projections in the cat as determined by the retrograde transport of horseradish peroxidase." Journal of Comparative Neurology **182**(1): 151-165.

Casey, K. (1969). "Somatic stimuli, spinal pathways, and size of cutaneous fibers influencing unit activity in the medial medullary reticular formation." Experimental neurology **25**(1): 35-56.

Casey, K. L. (1971). "Escape elicited by bulboreticular stimulation in the cat." International Journal of Neuroscience **2**(1): 29-34.

Casey, K. L. (1971). "Responses of bulboreticular units to somatic stimuli eliciting escape behavior in the cat." International Journal of Neuroscience **2**(1): 15-28.

Caterina, M. J., A. Leffler, A. Malmberg, W. Martin, J. Trafton, K. Petersen-Zeit, M. Koltzenburg, A. Basbaum and D. Julius (2000). "Impaired nociception and pain sensation in mice lacking the capsaicin receptor." science **288**(5464): 306-313.

Caterina, M. J., T. A. Rosen, M. Tominaga, A. J. Brake and D. Julius (1999). "A capsaicin-receptor homologue with a high threshold for noxious heat." Nature **398**(6726): 436-441.

Caterina, M. J., M. A. Schumacher, M. Tominaga, T. A. Rosen, J. D. Levine and D. Julius (1997). "The capsaicin receptor: a heat-activated ion channel in the pain pathway." Nature **389**(6653): 816-824.

Cavanaugh, D. J., A. T. Chesler, J. M. Bráz, N. M. Shah, D. Julius and A. I. Basbaum (2011). "Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons." The Journal of Neuroscience **31**(28): 10119-10127.

Cavanaugh, D. J., H. Lee, L. Lo, S. D. Shields, M. J. Zylka, A. I. Basbaum and D. J. Anderson (2009). "Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli." Proceedings of the National Academy of Sciences **106**(22): 9075-9080.

Cechetto, D. F., D. G. Standaert and C. B. Saper (1985). "Spinal and trigeminal dorsal horn projections to the parabrachial nucleus in the rat." Journal of Comparative Neurology **240**(2): 153-160.

Center, M. P. (2008). "Effectiveness of opioids in the treatment of chronic non-cancer pain." Pain Physician **11**: S181-S200.

Cesare, P. and P. McNaughton (1996). "A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin." Proceedings of the National Academy of Sciences **93**(26): 15435-15439.

Chandler, M. J., U.-T. Oh, S. F. Hobbs and R. D. Foreman (1994). "Responses of feline raphespinal neurons to urinary bladder distension." Journal of the autonomic nervous system **47**(3): 213-224.

Chaplan, S., F. Bach, J. Pogrel, J. Chung and T. Yaksh (1994). "Quantitative assessment of tactile allodynia in the rat paw." Journal of neuroscience methods **53**(1): 55-63.

Chapman, V., A. H. Dickenson and A. Tjølsen (1994). "Bi-directional effects of intrathecal NMDA and substance P on rat dorsal horn neuronal responses." Neuroscience letters **178**(1): 90-94.

Chapman, V., R. Suzuki, H. L. Chamarette, L. J. Rygh and A. H. Dickenson (1998). "Effects of systemic carbamazepine and gabapentin on spinal neuronal responses in spinal nerve ligated rats." Pain **75**(2): 261-272.

Chappell, A. S., M. J. Ossanna, H. Liu-Seifert, S. Iyengar, V. Skljarevski, L. C. Li, R. M. Bennett and H. Collins (2009). "Duloxetine, a centrally acting analgesic, in the treatment of patients with osteoarthritis knee pain: a 13-week, randomized, placebo-controlled trial." Pain **146**(3): 253-260.

Charan, J. and N. Kantharia (2013). "How to calculate sample size in animal studies?" Journal of Pharmacology and Pharmacotherapeutics **4**(4): 303.

Chen, J., S. K. Joshi, S. DiDomenico, R. J. Perner, J. P. Mikusa, D. M. Gauvin, J. A. Segreti, P. Han, X.-F. Zhang and W. Niforatos (2011). "Selective blockade of TRPA1 channel attenuates pathological pain without altering noxious cold sensation or body temperature regulation." Pain **152**(5): 1165-1172.

Chen, Y., M. a. Oatway and L. C. Weaver (2009). "Blockade of the 5-HT₃ receptor for days causes sustained relief from mechanical allodynia following spinal cord injury." Journal of neuroscience research **87**: 418-424.

Cheung, S. W., S. S. Nagarajan, P. H. Bedenbaugh, C. E. Schreiner, X. Wang and A. Wong (2001). "Auditory cortical neuron response differences under isoflurane versus pentobarbital anesthesia." Hearing research **156**(1): 115-127.

Chizh, B. A., P. M. Headley and T. M. Tzschentke (2001). "NMDA receptor antagonists as analgesics: focus on the NR2B subtype." Trends in Pharmacological Sciences **22**(12): 636-642.

Christensen, A. P. and D. P. Corey (2007). "TRP channels in mechanosensation: direct or indirect activation?" Nature Reviews Neuroscience **8**(7): 510-521.

Chu, K. L., P. Chandran, S. K. Joshi, M. F. Jarvis, P. R. Kym and S. McGaraughty (2011). "TRPV1-related modulation of spinal neuronal activity and behavior in a rat model of osteoarthritic pain." Brain research **1369**: 158-166.

Cialdai, C., S. Giuliani, C. Valenti, M. Tramontana and C. A. Maggi (2009). "Effect of Intra-articular 4-(S)-Amino-5-(4-{4-[2, 4-dichloro-3-(2, 4-dimethyl-8-quinolyloxy)methyl] phenylsulfonamido}-tetrahydro-2H-4-pyran-2-yl)-piperazine-5-oxopentyl](trimethyl) ammonium chloride hydrochloride (MEN16132), a Kinin B₂ Receptor Antagonist, on Nociceptive Response in Monosodium Iodoacetate-Induced Experimental Osteoarthritis in Rats." Journal of Pharmacology and Experimental Therapeutics **331**(3): 1025-1032.

Clark, F. M. and H. K. Proudfit (1991). "The projection of locus coeruleus neurons to the spinal cord in the rat determined by anterograde tracing combined with immunocytochemistry." Brain Research **538**(2): 231-245.

Clark, F. M. and H. K. Proudfit (1991). "Projections of neurons in the ventromedial medulla to pontine catecholamine cell groups involved in the modulation of nociception." Brain research **540**(1): 105-115.

Clark, F. M. and H. K. Proudfit (1993). "The projections of noradrenergic neurons in the A5 catecholamine cell group to the spinal cord in the rat: anatomical evidence that A5 neurons modulate nociception." Brain research **616**(1): 200-210.

Cleary, D. R. and M. M. Heinricher (2013). "Adaptations in responsiveness of brainstem pain-modulating neurons in acute compared with chronic inflammation." PAIN® **154**(6): 845-855.

Cleary, D. R., M. J. Neubert and M. M. Heinricher (2008). "Are opioid-sensitive neurons in the rostral ventromedial medulla inhibitory interneurons?" Neuroscience **151**(2): 564-571.

Clements, K. M., a. D. Ball, H. B. Jones, S. Brinckmann, S. J. Read and F. Murray (2009). "Cellular and histopathological changes in the infrapatellar fat pad in the moniodoacetate model of osteoarthritis pain." Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society **17**: 805-812.

Combe, R., S. Bramwell and M. J. Field (2004). "The monosodium iodoacetate model of osteoarthritis: a model of chronic nociceptive pain in rats?" Neuroscience letters **370**: 236-240.

Combe, R., S. Bramwell and M. J. Field (2004). "The monosodium iodoacetate model of osteoarthritis: a model of chronic nociceptive pain in rats?" Neuroscience letters **370**(2): 236-240.

Conaghan, P. G., J. Dickson and R. L. Grant (2008). "Care and management of osteoarthritis in adults: summary of NICE guidance." Bmj **336**(7642): 502-503.

Cook, A. J., C. J. Woolf, P. D. Wall and S. B. McMahon (1987). "Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input." Nature **325**(6100): 151-153.

Craig, A. (2003). "Pain mechanisms: labeled lines versus convergence in central processing." Annual Review of Neuroscience **26**(1): 1-30.

Craig, A., B. Heppelmann and H. G. Schaible (1988). "The projection of the medial and posterior articular nerves of the cat's knee to the spinal cord." The Journal of comparative neurology **276**(2): 279-288.

Craig, a. D. B. (2003). "Pain mechanisms: labeled lines versus convergence in central processing." Annual review of neuroscience **26**: 1-30.

Creamer, P., M. Hunt and P. Dieppe (1996). "Pain mechanisms in osteoarthritis of the knee: effect of intraarticular anesthetic." The Journal of Rheumatology **23**(6): 1031.

Cross III, W. W., K. J. Saleh, T. J. Wilt and R. L. Kane (2006). "Agreement about indications for total knee arthroplasty." Clinical orthopaedics and related research **446**: 34.

Cross, M., E. Smith, D. Hoy, S. Nolte, I. Ackerman, M. Fransen, L. Bridgett, S. Williams, F. Guillemin and C. L. Hill (2014). "The global burden of hip and knee osteoarthritis: estimates from the Global Burden of Disease 2010 study." Annals of the rheumatic diseases: annrheumdis-2013-204763.

D'Mello, R. and a. H. Dickenson (2008). "Spinal cord mechanisms of pain." British journal of anaesthesia **101**: 8-16.

D'Agostino, M. A., P. Conaghan, M. Le Bars, G. Baron, W. Grassi, E. Martin-Mola, R. Wakefield, J.-L. Brasseur, A. So and M. Backhaus (2005). "EULAR report on the use of ultrasonography in painful knee osteoarthritis. Part 1: prevalence of inflammation in osteoarthritis." Annals of the rheumatic diseases **64**(12): 1703-1709.

Da Silva, L. F., J. M. DeSantana and K. A. Sluka (2010). "Activation of NMDA receptors in the brainstem, rostral ventromedial medulla, and nucleus reticularis gigantocellularis mediates mechanical hyperalgesia produced by repeated intramuscular injections of acidic saline in rats." The journal of pain **11**(4): 378-387.

Danziger, N., J. Weil-Fugazza, D. Le Bars and D. Bouhassira (1999). "Alteration of descending modulation of nociception during the course of monoarthritis in the rat." The Journal of neuroscience : the official journal of the Society for Neuroscience **19**: 2394-2400.

Danziger, N., J. Weil - Fugazza, D. Le Bars and D. Bouhassira (2001). "Stage - dependent changes in the modulation of spinal nociceptive neuronal activity during the course of inflammation." European Journal of Neuroscience **13**(2): 230-240.

De Felice, M., R. Sanoja, R. Wang, L. Vera-Portocarrero, J. Oyarzo, T. King, M. H. Ossipov, T. W. Vanderah, J. Lai, G. O. Dussor, H. L. Fields, T. J. Price and F. Porreca (2011). "Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain." Pain **152**: 2701-2709.

De Koninck, Y. and J. L. Henry (1991). "Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation." Proceedings of the National Academy of Sciences **88**(24): 11344.

Delmas, P. and B. Coste (2013). "Mechano-gated ion channels in sensory systems." Cell **155**(2): 278-284.

Denoble, A. E., K. M. Huffman, T. V. Stabler, S. J. Kelly, M. S. Hershfield, G. E. McDaniel, R. E. Coleman and V. B. Kraus (2011). "Uric acid is a danger signal of increasing risk for osteoarthritis through inflammasome activation." Proceedings of the National Academy of Sciences **108**(5): 2088-2093.

Dequeker, J., S. Boonen, J. Aerssens and R. Westhovens (1996). "INVERSE RELATIONSHIP OSTEOARTHRITIS—OSTEOPOROSIS: WHAT IS THE EVIDENCE? WHAT ARE THE CONSEQUENCES?" Rheumatology **35**(9): 813-818.

Derkach, V., a. Barria and T. R. Soderling (1999). "Ca²⁺/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors." Proceedings of the National Academy of Sciences of the United States of America **96**: 3269-3274.

Dhaka, A., T. J. Earley, J. Watson and A. Patapoutian (2008). "Visualizing Cold Spots: TRPM8-Expressing Sensory Neurons and Their Projections." The Journal of Neuroscience **28**(3): 566-575.

Dhaka, A., V. Viswanath and A. Patapoutian (2006). "Trp ion channels and temperature sensation." Annu. Rev. Neurosci. **29**: 135-161.

Dib-Hajj, S., A. Rush, T. Cummins, F. Hisama, S. Novella, L. Tyrrell, L. Marshall and S. Waxman (2005). "Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons." Brain **128**(8): 1847-1854.

Dib-Hajj, S. D., T. R. Cummins, J. A. Black and S. G. Waxman (2010). "Sodium channels in normal and pathological pain." Annual review of neuroscience **33**: 325-347.

Dickenson, A. and A. F. Sullivan (1987). "Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation." Neuropharmacology **26**(8): 1235-1238.

Ding, J., Y. Xiao, D. Lu, Y.-R. Du, X.-Y. Cui and J. Chen (2011). "Effects of SKF-96365, a TRPC inhibitor, on melittin-induced inward current and intracellular Ca²⁺ rise in primary sensory cells." Neuroscience bulletin **27**(3): 135-142.

Dixon, W. J. (1980). "Efficient analysis of experimental observations." Annual review of pharmacology and toxicology **20**(1): 441-462.

Dogrul, A., M. H. Ossipov and F. Porreca (2009). "Differential mediation of descending pain facilitation and inhibition by spinal 5HT-3 and 5HT-7 receptors." Brain research **1280**: 52-59.

Doly, S., J. Fischer, M. J. Brisorgueil, D. Vergé and M. Conrath (2005). "Pre - and postsynaptic localization of the 5-HT₇ receptor in rat dorsal spinal cord: Immunocytochemical evidence." Journal of Comparative Neurology **490**(3): 256-269.

Dorn, T., H.-G. Schaible and R. F. Schmidt (1991). "Response properties of thick myelinated group II afferents in the medial articular nerve of normal and inflamed knee joints of the cat." Somatosensory & motor research **8**(2): 127-136.

Dougados, M., A. Gueguen, M. Nguyen, L. Berdah, M. Lequesne, B. Mazieres and E. Vignon (1996). "Radiological progression of hip osteoarthritis: definition, risk factors and correlations with clinical status." Annals of the rheumatic diseases **55**(6): 356-362.

Dowd, E., D. McQueen, I. Chessell and P. Humphrey (1998). "P2X receptor - mediated excitation of nociceptive afferents in the normal and arthritic rat knee joint." British journal of pharmacology **125**(2): 341-346.

Dubin, A. E., M. Schmidt, J. Mathur, M. J. Petrus, B. Xiao, B. Coste and A. Patapoutian (2012). "Inflammatory signals enhance piezo2-mediated mechanosensitive currents." Cell reports **2**(3): 511-517.

Duggan, A., I. Hendry, C. Morton, W. Hutchinson and Z. Zhao (1988). "Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat." Brain research **451**(1): 261-273.

Dunham, J., S. Hoedt-Schmidt and D. Kalbhen (1992). "Structural and metabolic changes in articular cartilage induced by iodoacetate." International journal of experimental pathology **73**(4): 455.

Dussor, G., M. J. Zylka, D. J. Anderson and E. W. McCleskey (2008). "Cutaneous sensory neurons expressing the Mrgprd receptor sense extracellular ATP and are putative nociceptors." Journal of neurophysiology **99**(4): 1581-1589.

Ebersberger, A., B. Grubb, H. Willingale, N. Gardiner, J. Nebe and H. G. Schaible (1999). "The intraspinal release of prostaglandin E₂ in a model of acute arthritis is accompanied by an up-regulation of cyclo-oxygenase-2 in the spinal cord." Neuroscience **93**(2): 775-781.

Edoff, K., M. Grenegard and C. Hildebrand (2000). "Retrograde tracing and neuropeptide immunohistochemistry of sensory neurones projecting to the cartilaginous distal femoral epiphysis of young rats." Cell and tissue research **299**(2): 193-200.

Eijkelkamp, N., J. Linley, J. Torres, L. Bee, A. Dickenson, M. Gringhuis, M. Minett, G. Hong, E. Lee and U. Oh (2013). "A role for Piezo2 in EPAC1-dependent mechanical allodynia." Nature communications **4**: 1682.

Elg, S., F. Marmigere, J. P. Mattsson and P. Ernfors (2007). "Cellular subtype distribution and developmental regulation of TRPC channel members in the mouse dorsal root ganglion." The Journal of comparative neurology **503**(1): 35-46.

Ellrich, J., C. Ulucan and C. Schnell (2000). "Are 'neutral cells' in the rostral ventromedial medulla subtypes of on-and off-cells?" Neuroscience research **38**(4): 419-423.

Ellrich, J., C. Ulucan and C. Schnell (2001). "Is the response pattern of on-and off-cells in the rostral ventromedial medulla to noxious stimulation independent of stimulation site?" Experimental brain research **136**(3): 394-399.

Engberg, I. and R. Ryall (1966). "The inhibitory action of noradrenaline and other monoamines on spinal neurones." The Journal of physiology **185**(2): 298-322.

England, S., S. Bevan and R. Docherty (1996). "PGE2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurones via the cyclic AMP-protein kinase A cascade." The Journal of Physiology **495**(Pt 2): 429-440.

Excellence, N. I. f. H. a. C. (2014). Management of osteoarthritis. NICE Pathways.

Excellence, N. I. o. H. a. C. (2014, February 2014). "NICE guidelines [CG177]." Osteoarthritis: Care and management in adults Retrieved October, 2014.

- Eyre, D. R. (2004). "Collagens and cartilage matrix homeostasis." Clinical orthopaedics and related research **427**: S118-S122.
- Fava, G. A. and E. Offidani (2011). "The mechanisms of tolerance in antidepressant action." Progress in Neuro-Psychopharmacology and Biological Psychiatry **35**(7): 1593-1602.
- Feldmann, M. and R. N. Maini (2003). "TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases." Nature Medicine **9**(10): 1245-1250.
- Felice, M. D., R. Sanoja, R. Wang, L. Vera-portocarrero, J. Oyarzo, T. King, M. H. Ossipov, T. W. Vanderah, J. Lai, G. O. Dussor, H. L. Fields, T. J. Price and F. Porreca (2011). "Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain." PAIN: 0-8.
- Felson, D. T. (1990). The epidemiology of knee osteoarthritis: results from the Framingham Osteoarthritis Study. Seminars in arthritis and rheumatism, Elsevier.
- Felson, D. T., K. D. Gross, M. C. Nevitt, M. Yang, N. E. Lane, J. C. Torner, C. E. Lewis and M. V. Hurley (2009). "The effects of impaired joint position sense on the development and progression of pain and structural damage in knee osteoarthritis." Arthritis Care & Research **61**(8): 1070-1076.
- Felson, D. T. and T. Neogi (2004). "Osteoarthritis: is it a disease of cartilage or of bone?" Arthritis & Rheumatism **50**(2): 341-344.
- Ferland, C. E., S. Laverty, F. Beaudry and P. Vachon (2011). "Gait analysis and pain response of two rodent models of osteoarthritis." Pharmacology, biochemistry, and behavior **97**: 603-610.
- Fernandez-Madrid, F., R. L. Karvonen, R. A. Teitge, P. R. Miller and W. G. Negendank (1994). "MR features of osteoarthritis of the knee." Magnetic resonance imaging **12**(5): 703-709.
- Fernihough, J., C. Gentry, S. Bevan and J. Winter (2005). "Regulation of calcitonin gene-related peptide and TRPV1 in a rat model of osteoarthritis." Neuroscience letters **388**(2): 75-80.
- Fernihough, J., C. Gentry, M. Malcangio, A. Fox, J. Rediske, T. Pellas, B. Kidd, S. Bevan and J. Winter (2004). "Pain related behaviour in two models of osteoarthritis in the rat knee." Pain **112**: 83-93.
- Ferreira - Gomes, J., S. Adaes, J. Sarkander and J. M. Castro - Lopes (2010). "Phenotypic alterations of neurons that innervate osteoarthritic joints in rats." Arthritis & Rheumatism **62**(12): 3677-3685.
- Fertleman, C. R., M. D. Baker, K. A. Parker, S. Moffatt, F. V. Elmslie, B. Abrahamsen, J. Ostman, N. Klugbauer, J. N. Wood and R. M. Gardiner (2006). "SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes." Neuron **52**(5): 767-774.
- Fields, H., J. Bry, I. Hentall and G. Zorman (1983). "The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat." The Journal of neuroscience **3**(12): 2545-2552.
- Fields, H., H. Vanegas, I. Hentall and G. Zorman (1983). "Evidence that disinhibition of brain stem neurones contributes to morphine analgesia."
- Fields, H. L., A. I. Basbaum, C. H. Clanton and S. D. Anderson (1977). "Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons." Brain research **126**(3): 441-453.
- Fields, H. L., M. M. Heinricher and P. Mason (1991). "Neurotransmitters in nociceptive modulatory circuits." Annual review of neuroscience **14**(1): 219-245.

Fields, H., A. Basbaum; and M. Heinricher; (2006). Wall and Melzack's Textbook of Pain. London, Elsevier Churchill Livingstone.

Finan, P. H., L. F. Buenaver, S. C. Bounds, S. Hussain, R. J. Park, U. J. Haque, C. M. Campbell, J. A. Haythornthwaite, R. R. Edwards and M. T. Smith (2013). "Discordance between pain and radiographic severity in knee osteoarthritis: findings from quantitative sensory testing of central sensitization." Arthritis & Rheumatism **65**(2): 363-372.

Fortin, P. R., J. R. Penrod, A. E. Clarke, Y. St - Pierre, L. Joseph, P. Bélisle, M. H. Liang, D. Ferland, C. B. Phillips and N. Mahomed (2002). "Timing of total joint replacement affects clinical outcomes among patients with osteoarthritis of the hip or knee." Arthritis & Rheumatism **46**(12): 3327-3330.

Fransen, M., L. Bridgett, L. March, D. Hoy, E. Penserga and P. Brooks (2011). "The epidemiology of osteoarthritis in Asia." International journal of rheumatic diseases **14**(2): 113-121.

Fries, J. F., B. Bruce and S. Shoor (2012). "Osteoarthritis, exercise, and knee replacement." The Journal of rheumatology **39**(4): 669-671.

Galan, A., J. Laird and F. Cervero (2004). "In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons." Pain **112**(3): 315-323.

Gallelli, L., O. Galasso, D. Falcone, S. Southworth, M. Greco, V. Ventura, P. Romualdi, A. Corigliano, R. Terracciano and R. Savino (2013). "The effects of nonsteroidal anti-inflammatory drugs on clinical outcomes, synovial fluid cytokine concentration and signal transduction pathways in knee osteoarthritis. A randomized open label trial." Osteoarthritis and Cartilage **21**(9): 1400-1408.

Gao, K. and P. Mason (2000). "Serotonergic raphe magnus cells that respond to noxious tail heat are not ON or OFF cells." Journal of Neurophysiology **84**(4): 1719-1725.

Garber, K. (2011). "Fate of novel painkiller mAbs hangs in balance." Nature biotechnology **29**(3): 173-174.

Gardner, E. P., J. H. Martin and T. M. Jessell (2000). "The bodily senses." Principles of neural science **4**: 430-450.

Gassner, M., R. Ruscheweyh and J. Sandkühler (2009). "Direct excitation of spinal GABAergic interneurons by noradrenaline." PAIN® **145**(1): 204-210.

Gauriau, C. and J. F. Bernard (2004). "A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: the forebrain." Journal of Comparative Neurology **468**(1): 24-56.

Gencosmanoglu, B. E., M. Eryavuz and S. Dervisoglu (2001). "Effects of some nonsteroidal anti-inflammatory drugs on articular cartilage of rats in an experimental model of osteoarthritis." Research in experimental medicine. Zeitschrift für die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie **200**: 215-226.

Ghosh, P. and P. A. Cheras (2001). "Vascular mechanisms in osteoarthritis." Best Practice & Research Clinical Rheumatology **15**(5): 693-709.

Giamberardino, M. A. (2003). "Referred muscle pain/hyperalgesia and central sensitisation." Journal of Rehabilitation Medicine-Supplements(41): 85-88.

Gibson, S., J. Polak, S. Bloom, I. Sabate, P. Mulderry, M. Ghatei, G. McGregor, J. Morrison, J. Kelly and R. Evans (1984). "Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species." The Journal of Neuroscience **4**(12): 3101-3111.

Giesler, G. J., J. T. Katter and R. J. Dado (1994). "Direct spinal pathways to the limbic system for nociceptive information." Trends in neurosciences **17**(6): 244-250.

Gjerstad, J., A. Tjølsen, F. Svendsen and K. Hole (2000). "Inhibition of spinal nociceptive responses after intramuscular injection of capsaicin involves activation of noradrenergic and opioid systems." Brain research **859**(1): 132-136.

Glover, T., B. Goodin, A. Horgas, L. Kindler, C. King, K. Sibille, C. Peloquin, J. Riley, R. Staud and L. Bradley (2012). "Vitamin D, race, and experimental pain sensitivity in older adults with knee osteoarthritis." Arthritis & Rheumatism **64**(12): 3926-3935.

Goel, M., W. G. Sinkins and W. P. Schilling (2002). "Selective association of TRPC channel subunits in rat brain synaptosomes." Journal of Biological Chemistry **277**(50): 48303-48310.

Gold, M. S. and G. F. Gebhart (2010). "Nociceptor sensitization in pain pathogenesis." Nature medicine **16**: 1248-1257.

Gold, M. S., D. B. Reichling, M. J. Shuster and J. D. Levine (1996). "Hyperalgesic agents increase a tetrodotoxin-resistant Na⁺ current in nociceptors." Proceedings of the National Academy of Sciences **93**(3): 1108-1112.

Gold MS, W. D., Kim CS, Wang R, Treanor J, Porreca F, Lai J. (2003). "Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain." J Neurosci **23**(1): 158-166.

Goldring, M. B. and S. R. Goldring (2010). "Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis." Annals of the New York Academy of Sciences **1192**(1): 230-237.

Gonçalves, L., A. Almeida and A. Pertovaara (2007). "Pronociceptive changes in response properties of rostroventromedial medullary neurons in a rat model of peripheral neuropathy." European Journal of Neuroscience **26**(8): 2188-2195.

Graven-Nielsen, T. and L. Arendt-Nielsen (2003). "Induction and assessment of muscle pain, referred pain, and muscular hyperalgesia." Current pain and headache reports **7**(6): 443-451.

Graven-Nielsen, T. and L. Arendt-Nielsen (2010). "Assessment of mechanisms in localized and widespread musculoskeletal pain." Nature Reviews Rheumatology **6**(10): 599-606.

Green, G. M., L. Lyons and a. H. Dickenson (1998). "Alpha2-adrenoceptor antagonists enhance responses of dorsal horn neurones to formalin induced inflammation." European journal of pharmacology **347**: 201-204.

Green, G. M., J. Scarth and A. Dickenson (2000). "An excitatory role for 5-HT in spinal inflammatory nociceptive transmission ; state-dependent actions via dorsal horn 5-HT 3 receptors in the anaesthetized rat." Pain **89**: 81-88.

Grigg, P., H. G. Schaible and R. F. Schmidt (1986). "Mechanical sensitivity of group III and IV afferents from posterior articular nerve in normal and inflamed cat knee." Journal of Neurophysiology **55**(4): 635-643.

Grubb, B. D. (2004). "Activation of sensory neurons in the arthritic joint." Osteoarthritic joint pain: 28-48.

Guan, Y., W. Guo, S.-P. Zou, R. Dubner and K. Ren (2003). "Inflammation-induced upregulation of AMPA receptor subunit expression in brain stem pain modulatory circuitry." Pain **104**: 401-413.

Guan, Y., R. Terayama, R. Dubner and K. Ren (2002). "Plasticity in excitatory amino acid receptor-mediated descending pain modulation after inflammation." The Journal of pharmacology and experimental therapeutics **300**: 513-520.

Guerhazi, A., J. Niu, D. Hayashi, F. W. Roemer, M. Englund, T. Neogi, P. Aliabadi, C. E. McLennan and D. T. Felson (2012). "Prevalence of abnormalities in knees detected by MRI in adults without knee osteoarthritis: population based observational study (Framingham Osteoarthritis Study)." BMJ: British Medical Journal **345**.

Guerne, P. A., R. Terkeltaub, B. Zuraw and M. Lotz (1989). "Inflammatory microcrystals stimulate interleukin-6 production and secretion by human monocytes and synoviocytes." Arthritis & Rheumatism **32**(11): 1443-1452.

Guilbaud, G., J. Besson, J. Oliveras and M. Wyon-Maillard (1973). "Modifications of the firing rate of bulbar reticular units (nucleus gigantocellularis) after intra-arterial injection of bradykinin into the limbs." Brain research **63**: 131-140.

Guingamp, C., P. Gegout-Pottie, L. Philippe, B. Terlain, P. Netter and P. Gillet (1997). "Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry." Arthritis and rheumatism **40**: 1670-1679.

Guo, H. and L. Y. M. Huang (2001). "Alteration in the voltage dependence of NMDA receptor channels in rat dorsal horn neurones following peripheral inflammation." The Journal of physiology **537**(1): 115-123.

Guo, W., S. Zou, Y. Guan, T. Ikeda, M. Tal, R. Dubner and K. Ren (2002). "Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia." The Journal of neuroscience **22**(14): 6208-6217.

Gutierrez, T., A. Nackley, M. Neely, K. Freeman, G. Edwards and A. Hohmann (2003). "Effects of neurotoxic destruction of descending noradrenergic pathways on cannabinoid antinociception in models of acute and tonic nociception." Brain research **987**(2): 176-185.

Guzman, R. E., M. G. Evans, S. Bove, B. Morenko and K. Kilgore (2003). "Mono-iodoacetate-induced histologic changes in subchondral bone and articular cartilage of rat femorotibial joints: an animal model of osteoarthritis." Toxicologic Pathology **31**(6): 619-624.

Gwilym, S. E., J. R. Keltner, C. E. Warnaby, A. J. Carr, B. Chizh, I. Chessell and I. Tracey (2009). "Psychophysical and functional imaging evidence supporting the presence of central sensitization in a cohort of osteoarthritis patients." Arthritis and rheumatism **61**: 1226-1234.

Haberberger, R. V., N. Bernardini, M. Kress, P. Hartmann, K. S. Lips and W. Kummer (2004). "Nicotinic acetylcholine receptor subtypes in nociceptive dorsal root ganglion neurons of the adult rat." Autonomic Neuroscience **113**(1): 32-42.

Hägermark, Ö., T. Hökfelt and B. Pernow (1978). "Flare and itch induced by substance P in human skin." Journal of Investigative Dermatology **71**(4): 233-235.

Hall, B., A. Limaye and A. B. Kulkarni (2009). "Overview: generation of gene knockout mice." Current protocols in cell biology: 19.12. 11-19.12. 17.

Han, Z.-S., E.-T. Zhang and A. Craig (1998). "Nociceptive and thermoreceptive lamina I neurons are anatomically distinct." Nature neuroscience **1**(3): 218-225.

Hanesch, U., M. Pawlak and J. J. McDougall (2003). "Gabapentin reduces the mechanosensitivity of fine afferent nerve fibres in normal and inflamed rat knee joints." Pain **104**(1): 363-366.

- Hannan, M. T., J. J. Anderson, Y. Zhang, D. Levy and D. T. Felson (1993). "Bone mineral density and knee osteoarthritis in elderly men and women. The Framingham Study." Arthritis & Rheumatism **36**(12): 1671-1680.
- Harper, A. and S. Lawson (1985). "Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities." The Journal of Physiology **359**(1): 47-63.
- Harris, D. and J. Sinclair (1981). "Periaqueductal gray stimulation: effect on characterized nucleus gigantocellularis neurons in the rat." Experimental neurology **72**(3): 552-558.
- Hartmann, B., S. Ahmadi, P. A. Heppenstall, G. R. Lewin, C. Schott, T. Borchardt, P. H. Seeburg, H. U. Zeilhofer, R. Sprengel and R. Kuner (2004). "The AMPA receptor subunits GluR-A and GluR-B reciprocally modulate spinal synaptic plasticity and inflammatory pain." Neuron **44**(4): 637-650.
- Harvey, R. J., U. B. Depner, H. W. Sessler, S. Ahmadi, C. Heindl, H. Reinold, T. G. Smart, K. Harvey, B. Scholtz and O. M. Abo-Salem (2004). "GlyR $\alpha 3$: an essential target for spinal PGE2-mediated inflammatory pain sensitization." Science **304**(5672): 884.
- Harvey, V. L. and A. H. Dickenson (2009). "Behavioural and electrophysiological characterisation of experimentally induced osteoarthritis and neuropathy in C57Bl/6 mice." Molecular pain **5**: 18.
- Haseeb, A. and T. M. Haqqi (2013). "Immunopathogenesis of osteoarthritis." Clinical immunology **146**(3): 185-196.
- Hawker, G., L. Stewart, M. French, J. Cibere, J. Jordan, L. March, M. Suarez-Almazor and R. Gooberman-Hill (2008). "Understanding the pain experience in hip and knee osteoarthritis, An OARSI/OMERACT initiative." Osteoarthritis and Cartilage **16**(4): 415-422.
- Hawker, G. A. (2009). "Experiencing painful osteoarthritis: what have we learned from listening?" Current opinion in rheumatology **21**(5): 507.
- Hayashida, K.-i., C. M. Peters, S. Gutierrez and J. C. Eisenach (2012). "Depletion of endogenous noradrenaline does not prevent spinal cord plasticity following peripheral nerve injury." The Journal of Pain **13**(1): 49-57.
- Haywood, L., D. McWilliams, C. Pearson, S. Gill, A. Ganesan, D. Wilson and D. Walsh (2003). "Inflammation and angiogenesis in osteoarthritis." Arthritis & Rheumatism **48**(8): 2173-2177.
- Headley, P., A. Duggan and B. Griersmith (1978). "Selective reduction by noradrenaline and 5-hydroxytryptamine of nociceptive responses of cat dorsal horn neurones." Brain research **145**(1): 185-189.
- Hefti, F. F., A. Rosenthal, P. A. Walicke, S. Wyatt, G. Vergara, D. L. Shelton and A. M. Davies (2006). "Novel class of pain drugs based on antagonism of NGF." Trends in Pharmacological Sciences **27**(2): 85-91.
- Heinricher, M., M. Morgan and H. Fields (1992). "Direct and indirect actions of morphine on medullary neurons that modulate nociception." Neuroscience **48**(3): 533-543.
- Heinricher, M., M. Morgan, V. Tortorici and H. Fields (1994). "Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla." Neuroscience **63**(1): 279-288.
- Heinricher, M., J. Schouten and E. Jobst (2001). "Activation of brainstem N-methyl-D-aspartate receptors is required for the analgesic actions of morphine given systemically." Pain **92**(1): 129-138.

Heinricher, M., I. Tavares, J. Leith and B. Lumb (2009). "Descending control of nociception: Specificity, recruitment and plasticity." Brain research reviews **60**(1): 214-225.

Heinricher, M. M., N. M. Barbaro and H. L. Fields (1989). "Putative nociceptive modulating neurons in the rostral ventromedial medulla of the rat: firing of on- and off-cells is related to nociceptive responsiveness." Somatosensory & motor research **6**(4): 427-439.

Henrich, F., W. Magerl, T. Klein, W. Greffrath and R.-D. Treede (2015). "Capsaicin-sensitive C- and A-fibre nociceptors control long-term potentiation-like pain amplification in humans." Brain **138**(9): 2505-2520.

Herrero, J. F. and F. Cervero (1996). "Supraspinal influences on the facilitation of rat nociceptive reflexes induced by carrageenan monoarthritis." Neuroscience Letters **209**: 21-24.

Herrero, J. F., J. M. Laird and J. A. Lopez-Garcia (2000). "Wind-up of spinal cord neurones and pain sensation: much ado about something?" Progress in neurobiology **61**(2): 169-203.

Hirsch, S., S. Just, L. Corradini and H. Doods (2013). "The CGRP receptor antagonist BIBN4096BS peripherally alleviates inflammatory pain in rats." Pain.

Hochberg, M. C., M. Wohlreich, P. Gaynor, S. Hanna and R. Risser (2012). "Clinically relevant outcomes based on analysis of pooled data from 2 trials of duloxetine in patients with knee osteoarthritis." The Journal of rheumatology **39**(2): 352-358.

Hochman, J., A. Davis, J. Elkayam, L. Gagliese and G. Hawker (2013). "Neuropathic pain symptoms on the modified painDETECT correlate with signs of central sensitization in knee osteoarthritis." Osteoarthritis and Cartilage **21**(9): 1236-1242.

Hochman, J., L. Gagliese, A. Davis and G. Hawker (2011). "Neuropathic pain symptoms in a community knee OA cohort." Osteoarthritis and Cartilage **19**(6): 647-654.

Holden, J. and H. Proudfit (1998). "Enkephalin neurons that project to the A7 catecholamine cell group are located in nuclei that modulate nociception: ventromedial medulla." Neuroscience **83**(3): 929-947.

HomeOffice (2013). "Annual Statistics of Scientific Procedure on Living Animals".

Horváth, Á., V. Tékus, M. Boros, G. Pozsgai, B. Botz, É. Borbély, J. Szolcsányi, E. Pintér and Z. Helyes (2016). "Transient receptor potential ankyrin 1 (TRPA1) receptor is involved in chronic arthritis: in vivo study using TRPA1-deficient mice." Arthritis research & therapy **18**(1): 1.

Hu, H. J., Y. Carrasquillo, F. Karim, W. E. Jung, J. M. Nerbonne, T. L. Schwarz and R. W. Gereau IV (2006). "The kv4. 2 potassium channel subunit is required for pain plasticity." Neuron **50**(1): 89-100.

Huang, J., X. Zhang and P. A. McNaughton (2006). "Inflammatory pain: the cellular basis of heat hyperalgesia." Current neuropharmacology **4**(3): 197.

Hughes, D. I., D. T. Scott, A. J. Todd and J. S. Riddell (2003). "Lack of evidence for sprouting of A β afferents into the superficial laminae of the spinal cord dorsal horn after nerve section." The Journal of neuroscience **23**(29): 9491-9499.

Hughes, S., L. Hickey, R. Hulse, B. Lumb and A. Pickering (2013). "Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury." Pain **154**(9): 1680-1690.

Hunt, S. P. and P. W. Mantyh (2001). "The molecular dynamics of pain control." Nature Reviews Neuroscience **2**(2): 83-91.

Hunter, D. J., A. Guermazi, F. Roemer, Y. Zhang and T. Neogi (2013). "Structural correlates of pain in joints with osteoarthritis." Osteoarthritis and Cartilage **21**(9): 1170-1178.

Hunter, D. J., J. J. McDougall and F. J. Keefe (2008). "The symptoms of osteoarthritis and the genesis of pain." Rheumatic diseases clinics of North America **34**: 623-643.

Hurley, M. V. (1999). "The role of muscle weakness in the pathogenesis of osteoarthritis." Rheumatic Disease Clinics of North America **25**(2): 283-298.

Hurley, M. V., D. L. Scott, J. Rees and D. J. Newham (1997). "Sensorimotor changes and functional performance in patients with knee osteoarthritis." Annals of the Rheumatic Diseases **56**(11): 641-648.

Hylden, J. L., D. Hayashi, R. Dubner and G. J. Bennett (1986). "Physiology and morphology of the lamina I spinomesencephalic projection." Journal of Comparative Neurology **247**(4): 505-515.

Hylden, J. L., H. Hayashi and G. J. Bennett (1986). "Lamina I spinomesencephalic neurons in the cat ascend via the dorsolateral funiculi." Somatosensory research **4**(1): 31-41.

Hylden, J. L., R. L. Nahin, R. J. Traub and R. Dubner (1989). "Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms." Pain **37**(2): 229-243.

Hylden, J. L., D. A. Thomas, M. J. Iadarola, R. L. Nahin and R. Dubner (1991). "Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms." European journal of pharmacology **194**(2): 135-143.

Imamura, M., S. T. Imamura, H. H. Kazyama, R. A. Targino, W. T. Hsing, L. P. M. De Souza, M. M. Cutait, F. Fregni and G. L. Camanho (2008). "Impact of nervous system hyperalgesia on pain, disability, and quality of life in patients with knee osteoarthritis: a controlled analysis." Arthritis Care & Research **59**(10): 1424-1431.

Imbe, H., Y. Iwai-Liao and E. Senba (2005). "Stress-induced hyperalgesia: animal models and putative mechanisms." Frontiers in bioscience: a journal and virtual library **11**: 2179-2192.

Indo, Y., M. Tsuruta, Y. Hayashida, M. A. Karim, K. Ohta, T. Kawano, H. Mitsubuchi, H. Tonoki, Y. Awaya and I. Matsuda (1996). "Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis." Nature genetics **13**(4): 485-488.

Ishikawa, G., Y. Koya, H. Tanaka and Y. Nagakura (2015). "Long-term analgesic effect of a single dose of anti-NGF antibody on pain during motion without notable suppression of joint edema and lesion in a rat model of osteoarthritis." Osteoarthritis and Cartilage.

Ivanavicius, S. P., A. D. Ball, C. G. Heapy, F. R. Westwood, F. Murray and S. J. Read (2007). "Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation." Pain **128**: 272-282.

Ivanova, A., M. Signore, N. Caro, N. D. Greene, A. J. Copp and J. P. Martinez - Barbera (2005). "In vivo genetic ablation by Cre - mediated expression of diphtheria toxin fragment A." Genesis **43**(3): 129-135.

Janusz, M. J., E. B. Hookfin, S. a. Heitmeyer, J. F. Woessner, a. J. Freemont, J. a. Hoyland, K. K. Brown, L. C. Hsieh, N. G. Almstead, B. De, M. G. Natchus, S. Pikul and Y. O. Taiwo (2001). "Moderation of iodoacetate-induced experimental osteoarthritis in rats by matrix metalloproteinase inhibitors." Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society **9**: 751-760.

Jarvis, M. F., P. Honore, C.-C. Shieh, M. Chapman, S. Joshi, X.-F. Zhang, M. Kort, W. Carroll, B. Marron and R. Atkinson (2007). "A-803467, a potent and selective Nav1.8 sodium channel blocker, attenuates neuropathic and inflammatory pain in the rat." Proceedings of the National Academy of Sciences **104**(20): 8520-8525.

Jessica O'Neill*, S. S., Stephen B McMahon and Anthony H Dickenson (2015). "Human psychophysics and rodent spinal neurones exhibit peripheral and central mechanisms of inflammatory pain in the UVB and UVB heat rekindling models." Journal of Physiology.

Ji, R.-r. and Y. Kawasaki (2009). "Pain and Plasticity." Inflammation: 361-369.

Ji, R. R., K. Befort, G. J. Brenner and C. J. Woolf (2002). "ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity." The Journal of neuroscience **22**(2): 478-485.

Ji, R. R., T. Kohno, K. A. Moore and C. J. Woolf (2003). "Central sensitization and LTP: do pain and memory share similar mechanisms?" Trends in neurosciences **26**(12): 696-705.

Jimenez, P. A., S. S. Glasson, O. V. Trubetskoy and H. B. Haimes (1997). "Spontaneous osteoarthritis in Dunkin Hartley guinea pigs: histologic, radiologic, and biochemical changes." Comparative Medicine **47**(6): 598-601.

Jones, R., G. Rubin, F. Berenbaum and J. Scheiman (2008). "Gastrointestinal and cardiovascular risks of nonsteroidal anti-inflammatory drugs." The American journal of medicine **121**(6): 464-474.

Jones, S. and A. R. Light (1990). "Termination patterns of serotonergic medullary raphespinal fibers in the rat lumbar spinal cord: an anterograde immunohistochemical study." Journal of Comparative Neurology **297**(2): 267-282.

Jones, S. L. and G. F. Gebhart (1986). "Characterization of coeruleospinal inhibition of the nociceptive tail-flick reflex in the rat: Mediation by spinal α_2 -adrenoceptors." Brain Research **364**(2): 315-330.

Jones, S. L. and G. F. Gebhart (1988). "Inhibition of spinal nociceptive transmission from the midbrain, pons and medulla in the rat: activation of descending inhibition by morphine, glutamate and electrical stimulation." Brain research **460**: 281-296.

Jordan, J. M., C. G. Helmick, J. B. Renner, G. Luta, A. D. Dragomir, J. Woodard, F. Fang, T. A. Schwartz, A. E. Nelson and L. M. Abbate (2009). "Prevalence of hip symptoms and radiographic and symptomatic hip osteoarthritis in African Americans and Caucasians: the Johnston County Osteoarthritis Project." The Journal of rheumatology **36**(4): 809-815.

Jordan, K., N. Arden, M. Doherty, B. Bannwarth, J. Bijlsma, P. Dieppe, K. Gunther, H. Hauselmann, G. Herrero-Beaumont and P. Kaklamanis (2003). "EULAR Recommendations 2003: an evidence based approach to the management of knee

osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT)." Annals of the rheumatic diseases **62**(12): 1145-1155.

Kalbhen, D. (1987). "Chemical model of osteoarthritis--a pharmacological evaluation." The Journal of Rheumatology **14**: 130.

Kalff, K.-M., M. El Mouedden, J. van Egmond, J. Veening, L. Joosten, G. J. Scheffer, T. Meert and K. Vissers (2010). "Pre-treatment with capsaicin in a rat osteoarthritis model reduces the symptoms of pain and bone damage induced by monosodium iodoacetate." European journal of pharmacology **641**: 108-113.

Kamisaki, Y., T. Hamada, K. Maeda, M. Ishimura and T. Itoh (1993). "Presynaptic $\alpha 2$ adrenoceptors inhibit glutamate release from rat spinal cord synaptosomes." Journal of neurochemistry **60**(2): 522-526.

Kaneko, S., T. Satoh, J. Chiba, C. Ju, K. Inoue and J. Kagawa (2000). "Interleukin-6 and interleukin-8 levels in serum and synovial fluid of patients with osteoarthritis." Cytokines, cellular & molecular therapy **6**(2): 71-79.

Karashima, Y., K. Talavera, W. Everaerts, A. Janssens, K. Y. Kwan, R. Vennekens, B. Nilius and T. Voets (2009). "TRPA1 acts as a cold sensor in vitro and in vivo." Proceedings of the National Academy of Sciences **106**(4): 1273-1278.

Kato, G., T. Yasaka, T. Katafuchi, H. Furue, M. Mizuno, Y. Iwamoto and M. Yoshimura (2006). "Direct GABAergic and glycinergic inhibition of the substantia gelatinosa from the rostral ventromedial medulla revealed by in vivo patch-clamp analysis in rats." The Journal of neuroscience **26**(6): 1787-1794.

Kawasaki, Y., T. Kohno, Z. Y. Zhuang, G. J. Brenner, H. Wang, C. Van Der Meer, K. Befort, C. J. Woolf and R. R. Ji (2004). "Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization." The Journal of neuroscience **24**(38): 8310-8321.

Kayser, V., I. E. Elfassi, B. Aubel, M. Melfort, D. Julius, J. A. Gingrich, M. Hamon and S. Bourgoin (2007). "Mechanical, thermal and formalin-induced nociception is differentially altered in 5-HT 1A-/-, 5-HT 1B-/-, 5-HT 2A-/-, 5-HT 3A-/- and 5-HTT-/- knock-out male mice." Pain **130**(3): 235-248.

Kean, W. F., R. Kean and W. W. Buchanan (2004). "Review Osteoarthritis : symptoms , signs and source of pain." Measurement **12**: 3 - 31.

Kellgren, J. and J. Lawrence (1957). "Radiological assessment of osteo-arthritis." Annals of the Rheumatic Diseases **16**(4): 494-502.

Kelly, S., K. Dobson and J. Harris (2013). "Spinal nociceptive reflexes are sensitized in the monosodium iodoacetate model of osteoarthritis pain in the rat." Osteoarthritis and Cartilage **21**(9): 1327-1335.

Kelly, S., J. P. Dunham, F. Murray, S. Read, L. F. Donaldson and S. N. Lawson (2012). "Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat." Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society: 1-9.

Kerr, B. J., V. Souslova, S. B. McMahon and J. N. Wood (2001). "A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain." Neuroreport **12**(14): 3077-3080.

Kerstein, P. C., D. del Camino, M. M. Moran and C. L. Stucky (2009). "Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors." Molecular pain **5**(1): 19.

Khasabov, S. G., T. Brink, M. Schupp, J. Noack and D. A. Simone (2012). "Changes in response properties of rostral ventromedial medulla neurons during prolonged inflammation: modulation by neurokinin-1 receptors." Neuroscience **224**: 235-248.

Khasabov, S. G., P. Malecha, J. Noack, J. Tabakov, K. Okamoto, D. A. Bereiter and D. A. Simone (2015). "Activation of rostral ventromedial medulla neurons by noxious stimulation of cutaneous and deep craniofacial tissues." Journal of neurophysiology **113**(1): 14-22.

Khasabov, S. G., S. D. Rogers, J. R. Ghilardi, C. M. Peters, P. W. Mantyh and D. A. Simone (2002). "Spinal neurons that possess the substance P receptor are required for the development of central sensitization." The Journal of neuroscience **22**(20): 9086-9098.

Kidd, E., A. Laporte, X. Langlois, C.-M. Fattaccini, C. Doyen, M. Lombard, H. Gozlan and M. Hamon (1993). "5-HT 3 receptors in the rat central nervous system are mainly located on nerve fibres and terminals." Brain research **612**(1): 289-298.

Kilkenny, C., W. Browne, I. C. Cuthill, M. Emerson and D. G. Altman (2010). "Animal research: reporting in vivo experiments: the ARRIVE guidelines." British journal of pharmacology **160**(7): 1577-1579.

Kim, Y. S., Y. Chu, L. Han, M. Li, Z. Li, P. C. LaVinka, S. Sun, Z. Tang, K. Park and M. J. Caterina (2014). "Central terminal sensitization of TRPV1 by descending serotonergic facilitation modulates chronic pain." Neuron **81**(4): 873-887.

Kitamura, T., J. Yamada, H. Sato and K. Yamashita (1993). "Cells of origin of the spinoparabrachial fibers in the rat: A study with fast blue and WGA - HRP." Journal of Comparative Neurology **328**(3): 449-461.

Kitto, K. F., J. E. Haley and G. L. Wilcox (1992). "Involvement of nitric oxide in spinally mediated hyperalgesia in the mouse." Neuroscience Letters **148**(1-2): 1-5.

Klein, K., A. Aeschlimann, S. Jordan, R. Gay, S. Gay and H. Sprott (2012). "ATP induced brain-derived neurotrophic factor expression and release from osteoarthritis synovial fibroblasts is mediated by purinergic receptor P2X4." PloS one **7**(5): e36693.

Kohno, T., K. A. Moore, H. Baba and C. J. Woolf (2003). "Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn." The Journal of physiology **548**(1): 131-138.

Kosek, E. and G. Ordeberg (2000). "Lack of pressure pain modulation by heterotopic noxious conditioning stimulation in patients with painful osteoarthritis before, but not following, surgical pain relief." Pain **88**(1): 69-78.

Kullmann, D. M., A. Ruiz, D. M. Rusakov, R. Scott, A. Semyanov and M. C. Walker (2005). "Presynaptic, extrasynaptic and axonal GABA A receptors in the CNS: where and why?" Progress in biophysics and molecular biology **87**(1): 33-46.

Kuner, R. (2010). "Central mechanisms of pathological pain." Nature medicine **16**: 1258-1266.

Kung, L.-H., K. Gong, M. Adedoyin, J. Ng, A. Bhargava, P. T. Ohara and L. Jasmin (2013). "Evidence for glutamate as a neuroglial transmitter within sensory ganglia." PLoS One **8**(7): e68312.

- Kuraishi, Y., Y. Harada, S. Aratani, M. Satoh and H. Takagi (1983). "Separate involvement of the spinal noradrenergic and serotonergic systems in morphine analgesia: the differences in mechanical and thermal algesic tests." Brain research **273**(2): 245-252.
- Kuraishi, Y., Y. Harada and H. Takagi (1979). "Noradrenaline regulation of pain-transmission in the spinal cord mediated by α -adrenoceptors." Brain Research **174**(2): 333-336.
- Kwan, K. Y., A. J. Allchorne, M. A. Vollrath, A. P. Christensen, D.-S. Zhang, C. J. Woolf and D. P. Corey (2006). "TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction." Neuron **50**(2): 277-289.
- Kwiat, G. C. and A. I. Basbaum (1992). "The origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn in the rat." Somatosensory & motor research **9**(2): 157-173.
- Lagerström, M. C., K. Rogoz, B. Abrahamsen, A.-L. Lind, C. Ölund, C. Smith, J. A. Mendez, Å. Wallén-Mackenzie, J. N. Wood and K. Kullander (2011). "A sensory subpopulation depends on vesicular glutamate transporter 2 for mechanical pain, and together with substance P, inflammatory pain." Proceedings of the National Academy of Sciences **108**(14): 5789-5794.
- Lai, J., M. S. Gold, C.-S. Kim, D. Biana, M. H. Ossipov, J. C. Hunter and F. Porreca (2002). "Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1. 8." Pain **95**(1): 143-152.
- Laine, L., W. B. White, A. Rostom and M. Hochberg (2008). COX-2 selective inhibitors in the treatment of osteoarthritis. Seminars in arthritis and rheumatism, Elsevier.
- LaMotte, R. H., C. N. Shain, D. A. Simone and E. Tsai (1991). "Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms." Journal of Neurophysiology **66**(1): 190-211.
- Lane, N., L. Webster, S. Lu, M. Gray, F. Hefti and P. Walicke (2005). RN624 (anti-NGF) improves pain and function in subjects with moderate knee osteoarthritis: A phase I study. ARTHRITIS AND RHEUMATISM, WILEY-LISS DIV JOHN WILEY & SONS INC, 111 RIVER ST, HOBOKEN, NJ 07030 USA.
- Lane, N. E., T. J. Schnitzer, C. A. Birbara, M. Mokhtarani, D. L. Shelton, M. D. Smith and M. T. Brown (2010). "Tanezumab for the treatment of pain from osteoarthritis of the knee." New England Journal of Medicine **363**(16): 1521-1531.
- Larsson, M. and J. Broman (2008). "Translocation of GluR1-containing AMPA receptors to a spinal nociceptive synapse during acute noxious stimulation." The Journal of neuroscience **28**(28): 7084.
- Latremoliere, A. and C. J. Woolf (2009). "Central sensitization: a generator of pain hypersensitivity by central neural plasticity." The Journal of Pain **10**(9): 895-926.
- Laursen, R. J., T. Graven-Nielsen, T. S. Jensen and L. Arendt-Nielsen (1999). "The effect of compression and regional anaesthetic block on referred pain intensity in humans." Pain **80**(1): 257-263.
- Lawrence, R. C., D. T. Felson, C. G. Helmick, L. M. Arnold, H. Choi, R. A. Deyo, S. Gabriel, R. Hirsch, M. C. Hochberg and G. G. Hunder (2008). "Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part II." Arthritis & Rheumatism **58**(1): 26-35.

Lawson, S. (1996). ". Peptides and cutaneous polymodal nociceptor neurones." Progress in brain research **113**: 369-385.

Lawson, S., B. Crepps and E. Perl (1997). "Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea - pig." The Journal of physiology **505**(1): 177-191.

Lawson, S. and P. Waddell (1991). "Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons." The Journal of Physiology **435**: 41.

Lawson, S. N., M. J. Perry, E. Prabhakar and P. W. McCarthy (1993). "Primary sensory neurones: neurofilament, neuropeptides and conduction velocity." Brain research bulletin **30**(3): 239-243.

Lee, M. C., L. Zambreanu, D. K. Menon and I. Tracey (2008). "Identifying brain activity specifically related to the maintenance and perceptual consequence of central sensitization in humans." The Journal of neuroscience **28**(45): 11642-11649.

Lekan, H. A., S. M. Carlton and R. E. Coggeshall (1996). "Sprouting of A β fibers into lamina II of the rat dorsal horn in peripheral neuropathy." Neuroscience letters **208**(3): 147-150.

Leung, C. G. and P. Mason (1998). "Physiological survey of medullary raphe and magnocellular reticular neurons in the anesthetized rat." Journal of neurophysiology **80**(4): 1630-1646.

Li, D. P., S. R. Chen, Y. Z. Pan, A. I. Levey and H. L. Pan (2002). "Role of presynaptic muscarinic and GABAB receptors in spinal glutamate release and cholinergic analgesia in rats." The Journal of physiology **543**(3): 807-818.

Li, L., M. Rutlin, Victoria E. Abaira, C. Cassidy, L. Kus, S. Gong, Michael P. Jankowski, W. Luo, N. Heintz, H. R. Koerber, C. J. Woodbury and David D. Ginty (2011). "The Functional Organization of Cutaneous Low-Threshold Mechanosensory Neurons." Cell **147**: 1615-1627.

Li, P. and M. Zhuo (1998). "Silent glutamatergic synapses and nociception in mammalian spinal cord." Nature **393**(6686): 695-698.

Li, X., G. Gibson, J.-S. Kim, J. Kroin, S. Xu, A. J. Van Wijnen and H.-J. Im (2011). "MicroRNA-146a is linked to pain-related pathophysiology of osteoarthritis." Gene **480**(1): 34-41.

Light, A. R. and A. M. Kavookjian (1988). "Morphology and ultrastructure of physiologically identified substantia gelatinosa (lamina II) neurons with axons that terminate in deeper dorsal horn laminae (III-V)." Journal of Comparative Neurology **267**(2): 172-189.

Lim, J., K. Luscombe, P. Jones and S. White (2006). "The effect of preoperative symptom severity on functional outcome of total knee replacement—patients with the lowest preoperative scores achieve the lowest marks." The Knee **13**(3): 216-219.

Lingard, E. A., J. N. Katz, E. A. Wright and C. B. Sledge (2004). "Predicting the outcome of total knee arthroplasty." The Journal of Bone & Joint Surgery **86**(10): 2179-2186.

Little, C. and S. Zaki (2012). "What constitutes an “animal model of osteoarthritis”—the need for consensus?" Osteoarthritis and Cartilage **20**(4): 261-267.

Liu, M. and J. N. Wood (2011). "The roles of sodium channels in nociception: implications for mechanisms of neuropathic pain." Pain Medicine **12**(s3): S93-S99.

Liu, P., A. Okun, J. Ren, R. Guo, M. H. Ossipov, J. Xie, T. King and F. Porreca (2011). "Ongoing pain in the MIA model of osteoarthritis." Neuroscience Letters.

Liu, Q., S. Vrontou, F. L. Rice, M. J. Zylka, X. Dong and D. J. Anderson (2007). "Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch." Nature neuroscience **10**(8): 946-948.

Liu, R., M. O'Connell, K. Johnson, K. Pritzker, N. Mackman and R. Terkeltaub (2000). "Extracellular signal, α -regulated kinase 1/extracellular signal, α -regulated kinase 2 mitogen, α -activated protein kinase signaling and activation of activator protein 1 and nuclear factor κ B transcription factors play central roles in interleukin-8 expression stimulated by monosodium urate monohydrate and calcium pyrophosphate crystals in monocytic cells." Arthritis & Rheumatism **43**(5): 1145-1155.

Loeser, R. F., S. R. Goldring, C. R. Scanzello and M. B. Goldring (2012). "Osteoarthritis: a disease of the joint as an organ." Arthritis & Rheumatism **64**(6): 1697-1707.

Lohmander, L. S., P. M. Englund, L. L. Dahl and E. M. Roos (2007). "The long-term consequence of anterior cruciate ligament and meniscus injuries osteoarthritis." The American journal of sports medicine **35**(10): 1756-1769.

Lolignier, S., N. Eijkelkamp and J. Wood (2015). "Mechanical allodynia." Pflügers Archiv - European Journal of Physiology **467**(1): 133-139.

Lovell, J. A., S. L. Stuesse, W. L. Cruce and T. Crisp (2000). "Strain differences in neuropathic hyperalgesia." Pharmacology Biochemistry and Behavior **65**(1): 141-144.

Lovick, T. (1993). "The periaqueductal gray-rostral medulla connection in the defence reaction: efferent pathways and descending control mechanisms." Behavioural brain research **58**(1): 19-25.

Ltd., T. P. I. (2015). "Teva and Xenon Provide Update on TV-45070 Phase 2b Study in Osteoarthritis Pain."

Lu, Y. and E. R. Perl (2005). "Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II)." The Journal of neuroscience **25**(15): 3900-3907.

Maffulli, N., U. G. Longo, N. Gougoulas, D. Caine and V. Denaro (2011). "Sport injuries: a review of outcomes." British medical bulletin **97**(1): 47-80.

Magerl, W., P. N. Fuchs, R. A. Meyer and R.-D. Treede (2001). "Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia." Brain **124**(9): 1754-1764.

Maier, C., R. Baron, T. Tölle, A. Binder, N. Birbaumer, F. Birklein, J. Gierthmühlen, H. Flor, C. Geber and V. Hugel (2010). "Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes." Pain **150**(3): 439-450.

Mainero, C., W.-T. Zhang, A. Kumar, B. R. Rosen and A. G. Sorensen (2007). "Mapping the spinal and supraspinal pathways of dynamic mechanical allodynia in the human trigeminal system using cardiac-gated fMRI." Neuroimage **35**(3): 1201-1210.

Malcangio, M., M. S. Ramer, M. G. Jones and S. B. McMahon (2000). "Abnormal substance P release from the spinal cord following injury to primary sensory neurons." European Journal of Neuroscience **12**(1): 397-399.

Malinow, R., D. V. Madison and R. W. Tsien (1988). "Persistent protein kinase activity underlying long-term potentiation."

Malinow, R., H. Schulman and R. W. Tsien (1989). "Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP." Science **245**(4920): 862.

Malmberg, A. B., L. R. Hedley, J. R. Jasper, J. C. Hunter and A. I. Basbaum (2001). "Contribution of $\alpha 2$ receptor subtypes to nerve injury - induced pain and its regulation by dexmedetomidine." British journal of pharmacology **132**(8): 1827-1836.

Mancuso, C. A., C. S. Ranawat, J. M. Esdaile, N. A. Johanson and M. E. Charlson (1996). "Indications for total hip and total knee arthroplasties: results of orthopaedic surveys." The Journal of arthroplasty **11**(1): 34-46.

Manis, J. P. (2007). "Knock out, knock in, knock down—genetically manipulated mice and the Nobel Prize." New England Journal of Medicine **357**(24): 2426-2429.

Mapp, P., D. Sagar, S. Ashraf, J. Burston, S. Suri, V. Chapman and D. Walsh (2013). "Differences in structural and pain phenotypes in the sodium monoiodoacetate and meniscal transection models of osteoarthritis." Osteoarthritis and Cartilage **21**(9): 1336-1345.

Marinelli, S., C. W. Vaughan, S. A. Schnell, M. W. Wessendorf and M. J. Christie (2002). "Rostral ventromedial medulla neurons that project to the spinal cord express multiple opioid receptor phenotypes." The Journal of neuroscience **22**(24): 10847-10855.

Mason, L., R. A. Moore, S. Derry, J. E. Edwards and H. J. McQuay (2004). "Systematic review of topical capsaicin for the treatment of chronic pain." Bmj **328**(7446): 991.

Mason, P. (2012). "Medullary circuits for nociceptive modulation." Current opinion in neurobiology **22**(4): 640-645.

Matsuyama, K., Y. Ohta and S. Mori (1988). "Ascending and descending projections of the nucleus reticularis gigantocellularis in the cat demonstrated by the anterograde neural tracer, Phaseolus vulgaris leucoagglutinin (PHA-L)." Brain research **460**(1): 124-141.

McCarthy, G. M. and D. J. McCarty (1992). "Effect of topical capsaicin in the therapy of painful osteoarthritis of the hands." The Journal of rheumatology **19**(4): 604-607.

McCarthy, P. and S. Lawson (1989). "Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity." Neuroscience **28**(3): 745-753.

McDonnell, J., O. Finnerty and J. Laffey (2011). "Stellate ganglion blockade for analgesia following upper limb surgery." Anaesthesia **66**(7): 611-614.

McDougall, J. J., B. Andruski, N. Schuelert, B. Hallgrímsson and J. R. Matyas (2009). "Unravelling the relationship between age, nociception and joint destruction in naturally occurring osteoarthritis of Dunkin Hartley guinea pigs." Pain **141**: 222-232.

McGaraughty, S., K. L. Chu, R. J. Perner, S. Didomenico, M. E. Kort and P. R. Kym (2010). "TRPA1 modulation of spontaneous and mechanically evoked firing of spinal neurons in uninjured, osteoarthritic, and inflamed rats." Molecular pain **6**: 14.

McMahon, S. and M. Koltzenburg, Eds. (2006). Wall and Melzack Textbook of Pain. Inflammatory Mediators and Modulators of Pain. London, Elsevier Churchill Livingstone.

McMullan, S. and B. M. Lumb (2006). "Midbrain control of spinal nociception discriminates between responses evoked by myelinated and unmyelinated heat nociceptors in the rat." Pain **124**(1): 59-68.

McNamee, K. E., A. Burleigh, L. L. Gompels, M. Feldmann, S. J. Allen, R. O. Williams, D. Dawbarn, T. L. Vincent and J. J. Inglis (2010). "Treatment of murine osteoarthritis with TrkA5 reveals a pivotal role for nerve growth factor in non-inflammatory joint pain." Pain **149**(2): 386-392.

Mease, P. J., S. Hanna, E. P. Frakes and R. D. Altman (2011). "Pain mechanisms in osteoarthritis: understanding the role of central pain and current approaches to its treatment." The Journal of rheumatology **38**: 1546-1551.

Melzack, R. and P. D. Wall (1965). "Pain mechanisms: a new theory." Science **150**(699): 971-979.

Mendell, L. (1984). "Modifiability of spinal synapses." Physiological reviews **64**(1): 260.

Mense, S. (1994). "Referral of muscle pain: new aspects." APS Journal **3**(1): 1-9.

Miki, K., Q. Zhou, W. Guo, Y. Guan, R. Terayama, R. Dubner, K. Ren, N. P. Pedersen, C. W. Vaughan, M. J. Christie and J. Neurophysiol (2002). "Changes in Gene Expression and Neuronal Phenotype in Brain Stem Pain Modulatory Circuitry After Inflammation Changes in Gene Expression and Neuronal Phenotype in Brain Stem Pain Modulatory Circuitry After Inflammation." Journal of Neurophysiology **87**: 750-760.

Millan, M. J. (2002). "Descending control of pain." Progress in Neurobiology **66**(6): 355-474.

Minett, M. S., S. Falk, S. Santana-Varela, Y. D. Bogdanov, M. A. Nassar, A.-M. Heegaard and J. N. Wood (2014). "Pain without nociceptors? Nav1.7-independent pain mechanisms." Cell reports **6**(2): 301-312.

Minett, M. S., M. A. Nassar, A. K. Clark, G. Passmore, A. H. Dickenson, F. Wang, M. Malcangio and J. N. Wood (2012). "Distinct Nav1.7-dependent pain sensations require different sets of sensory and sympathetic neurons." Nature communications **3**: 791.

Miquel, M. C., M. Emerit, A. Nosjean, A. Simon, P. Rumajogee, M. J. Brisorgueil, E. Doucet, M. Hamon and D. Verge (2002). "Differential subcellular localization of the 5-HT₃ - A receptor subunit in the rat central nervous system." European Journal of Neuroscience **15**(3): 449-457.

Miraucourt, L. S., R. Dallel and D. L. Voisin (2007). "Glycine inhibitory dysfunction turns touch into pain through PKC γ interneurons." PLoS One **2**(11): e1116.

Mishra, S. K., S. M. Tisel, P. Orestes, S. K. Bhangoo and M. A. Hoon (2011). "TRPV1 - lineage neurons are required for thermal sensation." The EMBO journal **30**(3): 582-593.

Mogil, J. S. and S. E. Crager (2004). "What should we be measuring in behavioral studies of chronic pain in animals?" Pain **112**(1): 12-15.

Mogil, J. S., S. G. Wilson, K. Bon, S. E. Lee, K. Chung, P. Raber, J. O. Pieper, H. S. Hain, J. K. Belknap and L. Hubert (1999). "Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception." Pain **80**(1): 67-82.

Mohan, G., E. Perilli, J. S. Kuliwaba, J. M. Humphries, I. H. Parkinson and N. L. Fazzalari (2011). "Application of in vivo micro-computed tomography in the

temporal characterisation of subchondral bone architecture in a rat model of low-dose monosodium iodoacetate-induced osteoarthritis." Arthritis Res Ther **13**: R210.

Mohrland, J., D. McManus and G. Gebhart (1982). "Lesion in nucleus reticularis gigantocellularis: effect on the antinociception produced by micro-injection of morphine and focal electrical stimulation in the periaqueductal gray matter." Brain research **231**(1): 143-152.

Moilanen, L., M. Hämäläinen, E. Nummenmaa, P. Ilmarinen, R. Nieminen, L. Lehtimäki and E. Moilanen (2015). "MIA-induced inflammation and joint pain are reduced in TRPA1 deficient mice–Potential role for trpa1 in osteoarthritis." Osteoarthritis and Cartilage **23**: A259.

Mokha, S., J. McMillan and A. Iggo (1986). "Pathways mediating descending control of spinal nociceptive transmission from the nuclei locus coeruleus (LC) and raphe magnus (NRM) in the cat." Experimental brain research **61**(3): 597-606.

Mokha, S. S., J. a. McMillan and a. Iggo (1985). "Descending control of spinal nociceptive transmission. Actions produced on spinal multireceptive neurones from the nuclei locus coeruleus (LC) and raphe magnus (NRM)." Experimental brain research. Experimentelle Hirnforschung. Expérimentation cérébrale **58**: 213-226.

Molina, C. and J. F. Herrero (2006). "The influence of the time course of inflammation and spinalization on the antinociceptive activity of the alpha2-adrenoceptor agonist medetomidine." European journal of pharmacology **532**: 50-60.

Momin, A. and J. N. Wood (2008). "Sensory neuron voltage-gated sodium channels as analgesic drug targets." Current opinion in neurobiology **18**(4): 383-388.

Morenko, B. J., S. E. Bove, L. Chen, R. E. Guzman, P. Juneau, T. Bocan, G. K. Peter, R. Arora and K. S. Kilgore (2004). "In vivo micro computed tomography of subchondral bone in the rat after intra-articular administration of monosodium iodoacetate." Journal of the American Association for Laboratory Animal Science **43**(1): 39-43.

Moriyama, T., T. Higashi, K. Togashi, T. Iida, E. Segi, Y. Sugimoto, T. Tominaga, S. Narumiya and M. Tominaga (2005). "Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins." Molecular pain **1**(1): 3.

Morrow, T. J. and K. L. Casey (1983). "Suppression of bulboreticular unit responses to noxious stimuli by analgesic mesencephalic stimulation." Somatosensory research **1**(2): 151-168.

Mouraux, A., G. D. Iannetti and L. Plaghki (2010). "Low intensity intra-epidermal electrical stimulation can activate Aδ-nociceptors selectively." Pain **150**(1): 199-207.

Murphy, L., T. A. Schwartz, C. G. Helmick, J. B. Renner, G. Tudor, G. Koch, A. Dragomir, W. D. Kalsbeek, G. Luta and J. M. Jordan (2008). "Lifetime risk of symptomatic knee osteoarthritis." Arthritis Care & Research **59**(9): 1207-1213.

Nagase, H., S. Kumakura and K. Shimada (2012). "Establishment of a novel objective and quantitative method to assess pain-related behavior in monosodium iodoacetate-induced osteoarthritis in rat knee." Journal of pharmacological and toxicological methods **65**(1): 29-36.

Naim, M., R. C. Spike, C. Watt, S. A. Shehab and A. J. Todd (1997). "Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have

dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents." The Journal of neuroscience **17**(14): 5536-5548.

Nassar, M. A., A. Levato, L. C. Stirling and J. N. Wood (2005). "Neuropathic pain develops normally in mice lacking both Na." Molecular pain **1**: 24.

Nassar, M. A., L. C. Stirling, G. Forlani, M. D. Baker, E. A. Matthews, A. H. Dickenson and J. N. Wood (2004). "Nociceptor-specific gene deletion reveals a major role for Nav1. 7 (PN1) in acute and inflammatory pain." Proceedings of the national Academy of Sciences of the United States of America **101**(34): 12706-12711.

Natura, G., K.-J. Bär, A. Eitner, M. K. Boettger, F. Richter, S. Hensellek, A. Ebersberger, J. Leuchtweis, T. Maruyama and G. O. Hofmann (2013). "Neuronal prostaglandin E2 receptor subtype EP3 mediates antinociception during inflammation." Proceedings of the National Academy of Sciences **110**(33): 13648-13653.

Nelson, A. E., Y. M. Golightly, J. B. Renner, T. A. Schwartz, V. B. Kraus, C. G. Helmick and J. M. Jordan (2013). "Brief Report: Differences in multijoint symptomatic osteoarthritis phenotypes by race and sex: The Johnston County Osteoarthritis Project." Arthritis & Rheumatism **65**(2): 373-377.

Neubert, M. J., W. Kincaid and M. M. Heinricher (2004). "Nociceptive facilitating neurons in the rostral ventromedial medulla." Pain **110**: 158-165.

Neugebauer, V., T. Lücke and H.-G. Schaible (1993). "Differential effects of N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists on the responses of rat spinal neurons with joint input." Neuroscience letters **155**(1): 29-32.

Neugebauer, V., P. Rumenapp and H.-G. Schaible (1996). "Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn neurons during development of acute inflammation." Neuroscience **71**(4): 1095-1109.

Neugebauer, V. and H. G. Schaible (1990). "Evidence for a central component in the sensitization of spinal neurons with joint input during development of acute arthritis in cat's knee." Journal of Neurophysiology **64**(1): 299-311.

Neuhold, L. A., L. Killar, W. Zhao, M. L. A. Sung, L. Warner, J. Kulik, J. Turner, W. Wu, C. Billinghamurst and T. Meijers (2001). "Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice." Journal of Clinical Investigation **107**(1): 35-64.

Neumann, S., J. M. Braz, K. Skinner, I. J. Llewellyn-Smith and A. I. Basbaum (2008). "Innocuous, not noxious, input activates PKC γ interneurons of the spinal dorsal horn via myelinated afferent fibers." The Journal of Neuroscience **28**(32): 7936-7944.

Neumann, S., T. P. Doubell, T. Leslie and C. J. Woolf (1996). "Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons." Nature **384**(6607): 360-364.

Nevitt, M. C., L. Xu, Y. Zhang, L. Y. Lui, W. Yu, N. E. Lane, M. Qin, M. C. Hochberg, S. R. Cummings and D. T. Felson (2002). "Very low prevalence of hip osteoarthritis among Chinese elderly in Beijing, China, compared with whites in the United States: the Beijing osteoarthritis study." Arthritis & Rheumatism **46**(7): 1773-1779.

Nicholson, B. and S. Verma (2004). "Comorbidities in chronic neuropathic pain." Pain Medicine **5**(s1): S9-S27.

Noguchi, K., R. Dubner, M. De Leon, E. Senba and M. Ruda (1994). "Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons." Journal of neuroscience research **37**(5): 596-603.

Nolano, M., D. A. Simone, G. Wendelschafer-Crabb, T. Johnson, E. Hazen and W. R. Kennedy (1999). "Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation." Pain **81**(1): 135-145.

O'Brien, C., C. Woolf, M. Fitzgerald, R. Lindsay and C. Molander (1989). "Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint." Neuroscience **32**(2): 493-502.

O'Neill, J. (2014). The translational studies of pain: from spinal neurones to human perception, University College London.

O'Neill, J., C. Brock, A. Olesen, T. Andresen, M. Nilsson and A. Dickenson (2012). "Unravelling the mystery of capsaicin: a tool to understand and treat pain." Pharmacological reviews **64**(4): 939-971.

O'Neill, J., C. Brock, A. E. Olesen, T. Andresen, M. Nilsson and A. H. Dickenson (2012). "Unravelling the mystery of capsaicin: a tool to understand and treat pain." Pharmacological reviews **64**(4): 939-971.

O'Connor, C. J., H. A. Leddy, H. C. Benefield, W. B. Liedtke and F. Guilak (2014). "TRPV4-mediated mechanotransduction regulates the metabolic response of chondrocytes to dynamic loading." Proceedings of the National Academy of Sciences **111**(4): 1316-1321.

Oatway, M. A., Y. Chen and L. C. Weaver (2004). "The 5-HT 3 receptor facilitates at-level mechanical allodynia following spinal cord injury." Pain **110**(1): 259-268.

Ogbonna, A., A. Clark, C. Gentry, C. Hobbs and M. Malcangio (2012). "Pain - like behaviour and spinal changes in the monosodium iodoacetate model of osteoarthritis in C57Bl/6 mice." European Journal of Pain.

Ohtori, S., G. Inoue, S. Orita, M. Takaso, Y. Eguchi, N. Ochiai, S. Kishida, K. Kuniyoshi, Y. Aoki and T. Ishikawa (2013). "Efficacy of combination of meloxicam and pregabalin for pain in knee osteoarthritis." Yonsei medical journal **54**(5): 1253-1258.

Okamoto, M. and Y. Atsuta (2010). "Cartilage degeneration is associated with augmented chemically-induced joint pain in rats: a pilot study." Clinical Orthopaedics and Related Research **468**(5): 1423-1427.

Okamoto, M., H. Baba, P. Goldstein, H. Higashi, K. Shimoji and M. Yoshimura (2001). "Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury." The Journal of physiology **532**(1): 241-250.

Okun, A., P. Liu, P. Davis, J. Ren, B. Remeniuk, T. Brion, M. H. Ossipov, J. Xie, G. O. Dussor, T. King and F. Porreca (2012). "Afferent drive elicits ongoing pain in a model of advanced osteoarthritis." Pain: 1-10.

Oliveria, S. A., D. T. Felson, J. I. Reed, P. A. Cirillo and A. M. Walker (1995). "Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization." Arthritis & Rheumatism **38**(8): 1134-1141.

Oliveria, S. A., D. T. Felson, J. I. Reed, P. A. Cirillo and A. M. Walker (2005). "Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization." Arthritis & Rheumatism **38**(8): 1134-1141.

Orita, S., T. Ishikawa, M. Miyagi, N. Ochiai, G. Inoue, Y. Eguchi, H. Kamoda, G. Arai, T. Toyone and Y. Aoki (2011). "Pain-related sensory innervation in monoiodoacetate-induced osteoarthritis in rat knees that gradually develops

neuronal injury in addition to inflammatory pain." BMC musculoskeletal disorders **12**(1): 134.

Orita, S., T. Ishikawa, M. Miyagi, N. Ochiai, G. Inoue, Y. Eguchi, H. Kamoda, G. Arai, T. Toyone, Y. Aoki, T. Kubo, K. Takahashi and S. Ohtori (2011). "Pain-related sensory innervation in monoiodoacetate-induced osteoarthritis in rat knees that gradually develops neuronal injury in addition to inflammatory pain." BMC musculoskeletal disorders **12**: 134.

Ossipov, M. H., G. O. Dussor and F. Porreca (2010). "Central Modulation of Pain." Conflict **120**.

Oteo - Álvaro, Á., M. A. Ruiz - Ibán, X. Miguens, A. Stern, J. Villoria and I. Sánchez - Magro (2014). "High Prevalence of Neuropathic Pain Features in Patients with Knee Osteoarthritis: A Cross - Sectional Study." Pain Practice.

Pan, Y.-Z., D.-P. Li and H.-L. Pan (2002). "Inhibition of glutamatergic synaptic input to spinal lamina II neurons by presynaptic $\alpha 2$ -adrenergic receptors." Journal of neurophysiology **87**(4): 1938-1947.

Pan, Z. Z. (2007). "Mechanisms of Opioid Tolerance." Molecular Pain: 413-422.

Panneton, W. and H. Burton (1985). "Projections from the paratrigeminal nucleus and the medullary and spinal dorsal horns to the peribrachial area in the cat." Neuroscience **15**(3): 779-797.

Park, J. S., N. Voitenko, R. S. Petralia, X. Guan, J. T. Xu, J. P. Steinberg, K. Takamiya, A. Sotnik, O. Kopach and R. L. Huganir (2009). "Persistent inflammation induces GluR2 internalization via NMDA receptor-triggered PKC activation in dorsal horn neurons." The Journal of neuroscience **29**(10): 3206-3219.

Park, W., I. Masuda, A. Cardenal-Escarcena, D. Palmer and D. McCarty (1996). "Inorganic pyrophosphate generation from adenosine triphosphate by cell-free human synovial fluid." The Journal of rheumatology **23**(4): 665-671.

Parsons, S., M. Ingram, A. Clarke-Cornwell and D. Symmons (2011). "A Heavy Burden: the occurrence and impact of musculoskeletal conditions in the United Kingdom today."

Patel, S. (2012). "Neuronal mechanisms in rodent models of osteoarthritic and cancer induced bone pain." UCL Thesis.

Patel, S. (2012). Neuronal mechanisms in rodent models of osteoarthritic and cancer induced bone pain, University College London.

Payne, C. E., A. R. Brown, J. W. Theile, A. J. Loucif, A. J. Alexandrou, M. D. Fuller, J. H. Mahoney, B. M. Antonio, A. C. Gerlach and D. M. Printzenhoff (2015). "A novel selective and orally bioavailable Nav1. 8 channel blocker, PF - 01247324, attenuates nociception and sensory neuron excitability." British journal of pharmacology.

Pearl, G. S. and K. V. Anderson (1978). "Response patterns of cells in the feline caudal nucleus reticularis gigantocellularis after noxious trigeminal and spinal stimulation." Experimental neurology **58**(2): 231-241.

Pergolizzi, J. V., R. B. Raffa, R. Taylor, G. Rodriguez, S. Nalamachu and P. Langley (2013). "A Review of Duloxetine 60 mg Once - Daily Dosing for the Management of Diabetic Peripheral Neuropathic Pain, Fibromyalgia, and Chronic Musculoskeletal Pain Due to Chronic Osteoarthritis Pain and Low Back Pain." Pain Practice **13**(3): 239-252.

Pertovaara, A. (2006). "Noradrenergic pain modulation." Progress in neurobiology **80**(2): 53-83.

Pertovaara, A. (2013). "The noradrenergic pain regulation system: a potential target for pain therapy." European journal of pharmacology **716**(1): 2-7.

Pertovaara, A. and T. Tukeva (1989). "Effect of subcutaneous formalin treatment on responses to bulboreticular nociceptive neurons in the rat." Brain research bulletin **23**(6): 457-462.

Peter, M., E. L. V. der Kraan, M. Henk and L. Beuningen (1990). "Degenerative knee joint lesions in mice after a single intra-articular collagenase injection. A new model of osteoarthritis." J Exp Pathol **71**: 19-31.

Peters, C. M., K.-i. Hayashida, E. E. Ewan, K. Nakajima, H. Obata, Q. Xu, T. L. Yaksh and J. C. Eisenach (2010). "Lack of analgesic efficacy of spinal ondansetron on thermal and mechanical hypersensitivity following spinal nerve ligation in the rat." Brain research **1352**: 83-93.

Petitjean, H., S. A. Pawlowski, S. L. Fraine, B. Sharif, D. Hamad, T. Fatima, J. Berg, C. M. Brown, L.-Y. Jan and A. Ribeiro-da-Silva (2015). "Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury." Cell reports **13**(6): 1246-1257.

Pezet, S. and S. B. McMahon (2006). "Neurotrophins: mediators and modulators of pain." Annu. Rev. Neurosci. **29**: 507-538.

Polgar, E., D. Hughes, J. Riddell, D. Maxwell, Z. Puskar and A. Todd (2003). "Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain." Pain **104**(1): 229-239.

Polgár, E., T. C. Sardella, S. Y. Tiong, S. Locke, M. Watanabe and A. J. Todd (2013). "Functional differences between neurochemically defined populations of inhibitory interneurons in the rat spinal dorsal horn." PAIN® **154**(12): 2606-2615.

Pomonis, J. D., J. M. Boulet, S. L. Gottshall, S. Phillips, R. Sellers, T. Bunton and K. Walker (2005). "Development and pharmacological characterization of a rat model of osteoarthritis pain." Pain **114**(3): 339-346.

Pomonis, J. D., J. M. Boulet, S. L. Gottshall, S. Phillips, R. Sellers, T. Bunton and K. Walker (2005). "Development and pharmacological characterization of a rat model of osteoarthritis pain." Pain **114**: 339-346.

Pond, M. and G. Nuki (1973). "Experimentally-induced osteoarthritis in the dog." Annals of the Rheumatic Diseases **32**(4): 387-388.

Poole, A. R. (1993). "Cartilage in health and disease." Arthritis and allied conditions: a textbook of rheumatology **15**: 223-269.

Porreca, F., M. H. Ossipov and G. F. Gebhart (2002). "Chronic pain and medullary descending facilitation." Trends in neurosciences **25**: 319-325.

Potrebic, S., A. H. Ahn, K. Skinner, H. L. Fields and A. I. Basbaum (2003). "Peptidergic nociceptors of both trigeminal and dorsal root ganglia express serotonin 1D receptors: implications for the selective antimigraine action of triptans." The Journal of neuroscience **23**(34): 10988-10997.

Potrebic, S., H. Fields and P. Mason (1994). "Serotonin immunoreactivity is contained in one physiological cell class in the rat rostral ventromedial medulla." The Journal of neuroscience **14**(3): 1655-1665.

Power, J. D., A. V. Perruccio and E. M. Badley (2005). "Pain as a mediator of sleep problems in arthritis and other chronic conditions." Arthritis Care & Research **53**(6): 911-919.

Priestley, J., G. Michael, S. Averill, M. Liu and N. Willmott (2002). "Regulation of nociceptive neurons by nerve growth factor and glial cell line derived neurotrophic factor." Canadian journal of physiology and pharmacology **80**(5): 495-505.

Proudfit, H. K. and D. L. Hammond (1981). "Alterations in nociceptive threshold and morphine-induced analgesia produced by intrathecally administered amine antagonists." Brain Research **218**(1): 393-399.

Pulichino, A.-M., S. Rowland, T. Wu, P. Clark, D. Xu, M.-C. Mathieu, D. Riendeau and L. P. Audoly (2006). "Prostacyclin antagonism reduces pain and inflammation in rodent models of hyperalgesia and chronic arthritis." Journal of Pharmacology and Experimental Therapeutics **319**(3): 1043-1050.

Quick, K., J. Zhao, N. Eijkelkamp, J. E. Linley, F. Rugiero, J. J. Cox, R. Raouf, M. Gringhuis, J. E. Sexton and J. Abramowitz (2012). "TRPC3 and TRPC6 are essential for normal mechanotransduction in subsets of sensory neurons and cochlear hair cells." Open Biology **2**(5).

Rahman, W., C. S. Bauer, K. Bannister, J.-L. Vonsy, A. C. Dolphin and A. H. Dickenson (2009). "Descending serotonergic facilitation and the antinociceptive effects of pregabalin in a rat model of osteoarthritic pain." Molecular pain **5**: 45.

Rahman, W., R. D'Mello and A. H. Dickenson (2008). "Peripheral Nerve Injury-Induced Changes in Spinal α 2-Adrenoceptor-Mediated Modulation of Mechanically Evoked Dorsal Horn Neuronal Responses." The Journal of Pain **9**(4): 350-359.

Rahman, W. and A. Dickenson (2015). "Osteoarthritis-dependent changes in antinociceptive action of Na v 1.7 and Na v 1.8 sodium channel blockers: An in vivo electrophysiological study in the rat." Neuroscience **295**: 103-116.

Rahman, W., R. Suzuki, L. J. Rygh and A. H. Dickenson (2004). "Descending serotonergic facilitation mediated through rat spinal 5HT3 receptors is unaltered following carrageenan inflammation." Neuroscience letters **361**: 229-231.

Rahme, E., A. Barkun, H. Nedjar, S. Gaugris and D. Watson (2008). "Hospitalizations for upper and lower GI events associated with traditional NSAIDs and acetaminophen among the elderly in Quebec, Canada." The American journal of gastroenterology **103**(4): 872-882.

Rasanen P, P. P., Sintonen H, Koivisto A-M, Blom M, Ryyanen O-P, Chassin MR, Kahn KL, Merrick NJ, et al. (2007). "Effectiveness of hip or knee replacement surgery in terms of quality-adjusted life years and costs." Acta Orthop **78**: 108-115.

Rashid, M., Y. Theberge, S. Elmes, M. Perkins and F. McIntosh (2013). "Pharmacological validation of early and late phase of rat mono - iodoacetate model using the Tekscan system." European Journal of Pain **17**(2): 210-222.

Rashid, M. H., Y. Theberge, S. Elmes, M. Perkins and F. McIntosh (2012). "Pharmacological validation of early and late phase of rat mono - iodoacetate model using the Tekscan system." European Journal of Pain.

Ren, K. and R. Dubner (1996). "Enhanced descending modulation of nociception in rats with persistent hindpaw inflammation." Journal of neurophysiology **76**: 3025-3037.

Reynolds, D. V. (1969). "Surgery in the rat during electrical analgesia induced by focal brain stimulation." Science **164**(3878): 444.

Ribeiro - Da - Silva, A., P. Tagari and A. Cuello (1989). "Morphological characterization of substance P - like immunoreactive glomeruli in the superficial

dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: A quantitative study." Journal of Comparative Neurology **281**(4): 497-515.

Richardson, J. D. and M. R. Vasko (2002). "Cellular mechanisms of neurogenic inflammation." The Journal of pharmacology and experimental therapeutics **302**: 839-845.

Ritz, L. A. and J. D. Greenspan (1985). "Morphological features of lamina V neurons receiving nociceptive input in cat sacrocaudal spinal cord." Journal of Comparative Neurology **238**(4): 440-452.

Robinson, D. A., A. A. Calejesan and M. Zhuo (2002). "Long-lasting changes in rostral ventral medulla neuronal activity after inflammation." The Journal of Pain **3**(4): 292-300.

Roza, C., J. M. Laird, V. Souslova, J. N. Wood and F. Cervero (2003). "The tetrodotoxin-resistant Na⁺ channel Nav1. 8 is essential for the expression of spontaneous activity in damaged sensory axons of mice." The Journal of physiology **550**(3): 921-926.

Ryan, L., J. Rachow and D. McCarty (1991). "Synovial fluid ATP: a potential substrate for the production of inorganic pyrophosphate." The Journal of rheumatology **18**(5): 716-720.

Sagar, D. R., J. J. Burston, G. J. Hathway, S. G. Woodhams, R. G. Pearson, A. J. Bennett, D. A. Kendall, B. E. Scammell and V. Chapman (2011). "The contribution of spinal glial cells to chronic pain behaviour in the monosodium iodoacetate model of osteoarthritic pain." Mol Pain **7**: 88.

Sagar, D. R., L. Nwosu, D. A. Walsh and V. Chapman (2015). "Dissecting the contribution of knee joint NGF to spinal nociceptive sensitization in a model of OA pain in the rat." Osteoarthritis and Cartilage.

Sagar, D. R., L. E. Staniaszek, B. N. Okine, S. Woodhams, L. M. Norris, R. G. Pearson, M. J. Garle, S. P. Alexander, A. J. Bennett and D. A. Barrett (2010). "Tonic modulation of spinal hyperexcitability by the endocannabinoid receptor system in a rat model of osteoarthritis pain." Arthritis & Rheumatism **62**(12): 3666-3676.

Sagen, J. and H. K. Proudfit (1984). "Effect of intrathecally administered noradrenergic antagonists on nociception in the rat." Brain research **310**(2): 295-301.

Saito, T. and T. Koshino (2000). "Distribution of neuropeptides in synovium of the knee with osteoarthritis." Clinical orthopaedics and related research **376**: 172-182.

Sandkuhler, J. and G. Gebhart (1984). "Relative contributions of the nucleus raphe magnus and adjacent medullary reticular formation to the inhibition by stimulation in the periaqueductal gray of a spinal nociceptive reflex in the pentobarbital-anesthetized rat." Brain Research **305**(1): 77-87.

Sarzi-Putini, P., M. A. Cimmino, R. Scarpa, R. Caporali, F. Parazzini, A. Zaninelli, F. Atzeni and B. Canesi (2005). Osteoarthritis: an overview of the disease and its treatment strategies. Seminars in arthritis and rheumatism, Elsevier.

Schaible, H.-G. and F. Richter (2004). "Pathophysiology of pain." Langenbeck's Archives of Surgery **389**(4): 237-243.

Schaible, H.-G. and R. F. Schmidt "Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis."

Schaible, H. G. (2009). The Nociceptive System of the Joint.

Schaible, H. G., V. Neugebauer, F. Cervero and R. F. Schmidt (1991). "Changes in tonic descending inhibition of spinal neurons with articular input during the

development of acute arthritis in the cat." Journal of neurophysiology **66**: 1021-1032.

Schaible, H. G., F. Richter, A. Ebersberger, M. K. Boettger, H. Vanegas, G. Natura, E. Vazquez and G. Segond von Banchet (2009). "Joint pain." Experimental brain research **196**(1): 153-162.

Schaible, H. G., R. Schmidt and W. Willis (1987). "Convergent inputs from articular, cutaneous and muscle receptors onto ascending tract cells in the cat spinal cord." Experimental brain research **66**(3): 479-488.

Schaible, H. G. and R. F. Schmidt (1985). "Effects of an experimental arthritis on the sensory properties of fine articular afferent units." Journal of Neurophysiology **54**(5): 1109-1122.

Schaible, H. G. and R. F. Schmidt (1988). "Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis." Journal of Neurophysiology **60**(6): 2180-2195.

Schein, J. R., M. R. Kosinski, C. Janagap-Benson, K. Gajria, P. Lin and J. D. Freedman (2008). "Functionality and health-status benefits associated with reduction of osteoarthritis pain." Current Medical Research and Opinion **24**(5): 1255-1265.

Schnell, C., C. Ulucan and J. Ellrich (2002). "Atypical on-, off-and neutral cells in the rostral ventromedial medulla oblongata in rat." Experimental brain research **145**(1): 64-75.

Schnitzer, T., N. Lane, C. Birbara, M. Smith, S. Simpson and M. Brown (2011). "Long-term open-label study of tanezumab for moderate to severe osteoarthritic knee pain." Osteoarthritis and Cartilage **19**(6): 639-646.

Schnitzer, T., C. Morton and S. Coker (1994). Topical capsaicin therapy for osteoarthritis pain: achieving a maintenance regimen. Seminars in arthritis and rheumatism, Elsevier.

Schnitzer, T. J., E. F. Ekman, E. L. Spierings, H. S. Greenberg, M. D. Smith, M. T. Brown, C. R. West and K. M. Verburg (2014). "Efficacy and safety of tanezumab monotherapy or combined with non-steroidal anti-inflammatory drugs in the treatment of knee or hip osteoarthritis pain." Annals of the rheumatic diseases: annrheumdis-2013-204905.

Schoffnegger, D., R. Ruscheweyh and J. Sandkühler (2008). "Spread of excitation across modality borders in spinal dorsal horn of neuropathic rats." Pain **135**(3): 300-310.

Schuelert, N. and J. McDougall (2006). "Electrophysiological evidence that the vasoactive intestinal peptide receptor antagonist VIP₆₋₂₈ reduces nociception in an animal model of osteoarthritis." Osteoarthritis and cartilage **14**(11): 1155-1162.

Schuelert, N. and J. J. McDougall (2009). "Grading of monosodium iodoacetate-induced osteoarthritis reveals a concentration-dependent sensitization of nociceptors in the knee joint of the rat." Neuroscience letters **465**: 184-188.

Schuelert, N. and J. J. McDougall (2012). "Involvement of Nav 1.8 sodium ion channels in the transduction of mechanical pain in a rodent model of osteoarthritis." Arthritis Res Ther **14**(1): R5.

Scott, D. (2006). Osteoarthritis and Rheumatoid Arthritis. Wall and Melzack's Textbook of Pain. K. M. McMahon SB. London, Elsevier: 653-667.

Seagrove, L. C., R. Suzuki and A. H. Dickenson (2004). "Electrophysiological characterisations of rat lamina I dorsal horn neurones and the involvement of excitatory amino acid receptors." Pain **108**(1): 76-87.

Seal, R. P., X. Wang, Y. Guan, S. N. Raja, C. J. Woodbury, A. I. Basbaum and R. H. Edwards (2009). "Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors." Nature **462**(7273): 651-655.

Segal, M. and D. Sandberg (1977). "Analgesia produced by electrical stimulation of catecholamine nuclei in the rat brain." Brain research **123**(2): 369-372.

Sellam, J. and F. Berenbaum (2010). "The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis." Nature Reviews Rheumatology **6**(11): 625-635.

Shakoor, N., A. Agrawal and J. A. Block (2008). "Reduced lower extremity vibratory perception in osteoarthritis of the knee." Arthritis Care & Research **59**(1): 117-121.

Sharma, L. (1999). "Proprioceptive impairment in knee osteoarthritis." Rheumatic Disease Clinics of North America **25**(2): 299-314.

Sharma, L., C. Lou, D. T. Felson, D. D. Dunlop, G. Kirwan - Mellis, K. W. Hayes, D. Weinrach and T. S. Buchanan (1999). "Laxity in healthy and osteoarthritic knees." Arthritis & Rheumatism **42**(5): 861-870.

Shehab, S., R. Spike and A. Todd (2003). "Evidence against cholera toxin B subunit as a reliable tracer for sprouting of primary afferents following peripheral nerve injury." Brain research **964**(2): 218-227.

Shields, S. D., H. S. Ahn, Y. Yang, C. Han, R. P. Seal, J. N. Wood, S. G. Waxman and S. D. Dib-Hajj (2012). "Na^v1.8 expression is not restricted to nociceptors in mouse peripheral nervous system." Pain.

Shigemura, T., S. Ohtori, S. Kishida, J. Nakamura, M. Takeshita, M. Takazawa, Y. Harada and K. Takahashi (2011). "Neuropathic pain in patients with osteoarthritis of hip joint." European Orthopaedics and Traumatology **2**(3-4): 73-77.

Sikandar, S., K. Bannister and A. H. Dickenson (2012). "Brainstem facilitations and descending serotonergic controls contribute to visceral nociception but not pregabalin analgesia in rats." Neuroscience letters **519**(1): 31-36.

Sikandar, S. and A. H. Dickenson (2011). "Pregabalin modulation of spinal and brainstem visceral nociceptive processing." PAIN® **152**(10): 2312-2322.

Silva, M., D. Amorim, A. Almeida, I. Tavares, F. Pinto-Ribeiro and C. Morgado (2013). "Pronociceptive changes in the activity of rostroventromedial medulla (RVM) pain modulatory cells in the streptozotocin-diabetic rat." Brain research bulletin **96**: 39-44.

Silverman, J. D. and L. Kruger (1988). "Acid phosphatase as a selective marker for a class of small sensory ganglion cells in several mammals: spinal cord distribution, histochemical properties, and relation to fluoride-resistant acid phosphatase (FRAP) of rodents." Somatosensory research **5**(3): 219-246.

Silverman, J. D. and L. Kruger (1988). "Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations." Somatosensory research **5**(3): 259-267.

Skagerberg, G. and A. Björklund (1985). "Topographic principles in the spinal projections of serotonergic and non-serotonergic brainstem neurons in the rat." Neuroscience **15**(2): 445-480.

Skerratt, S. E. and C. W. West (2015). "Ion channel therapeutics for pain." Channels(just-accepted): 00-00.

Skyba, D., R. Radhakrishnan, J. Rohlwing, A. Wright and K. Sluka (2003). "Joint manipulation reduces hyperalgesia by activation of monoamine receptors but not opioid or GABA receptors in the spinal cord." Pain **106**(1): 159-168.

Smith, S. (2012). "OAnation 2012." arthritis care.

Snider, W. D. and S. B. McMahon (1998). "Tackling pain at the source: new ideas about nociceptors." Neuron **20**(4): 629-632.

So, K., K. Haraguchi, K. Asakura, K. Isami, S. Sakimoto, H. Shirakawa, Y. Mori, T. Nakagawa and S. Kaneko (2015). "Involvement of TRPM2 in a wide range of inflammatory and neuropathic pain mouse models." Journal of pharmacological sciences **127**(3): 237-243.

Sommer, C. (2004). "Serotonin in pain and analgesia." Molecular neurobiology **30**(2): 117-125.

Sommer, C. and M. Kress (2004). "Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia." Neuroscience Letters **361**(1-3): 184-187.

Sorge, R. E., L. J. Martin, K. A. Isbester, S. G. Sotocinal, S. Rosen, A. H. Tuttle, J. S. Wieskopf, E. L. Acland, A. Dokova and B. Kadoura (2014). "Olfactory exposure to males, including men, causes stress and related analgesia in rodents." Nature methods **11**(6): 629-632.

South, S. M., T. Kohno, B. K. Kaspar, D. Hegarty, B. Vissel, C. T. Drake, M. Ohata, S. Jenab, A. W. Sailer and S. Malkmus (2003). "A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain." The Journal of neuroscience **23**(12): 5031-5040.

Spassova, M. A., T. Hewavitharana, W. Xu, J. Soboloff and D. L. Gill (2006). "A common mechanism underlies stretch activation and receptor activation of TRPC6 channels." Proceedings of the National Academy of Sciences **103**(44): 16586-16591.

Spector, T. D. and A. J. MacGregor (2004). "Risk factors for osteoarthritis: genetics." Osteoarthritis and cartilage **12**: 39-44.

Spierings, E. L., J. Fidelholtz, G. Wolfram, M. D. Smith, M. T. Brown and C. R. West (2013). "A phase III placebo-and oxycodone-controlled study of tanezumab in adults with osteoarthritis pain of the hip or knee." Pain **154**(9): 1603-1612.

Spike, R., Z. Puskar, D. Andrew and A. Todd (2003). "A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord." European Journal of Neuroscience **18**(9): 2433-2448.

Spinella, M., M. Cooper and R. Bodnar (1996). "Excitatory amino acid antagonists in the rostral ventromedial medulla inhibit mesencephalic morphine analgesia in rats." Pain **64**(3): 545.

Stanfa, L. C. and a. H. Dickenson (1994). Enhanced alpha-2 adrenergic controls and spinal morphine potency in inflammation. Neuroreport. **5**: 469-472.

Stephen McMahon, M. K., Ed. (2006). Wall and Melzack's Textbook of Pain. Chapter 5: Plasticity and Pain - role of the dorsal horn. London, Elsevier Churchill Livingstone.

Stevenson, G. W., H. Mercer, J. Cormier, C. Dunbar, L. Benoit, C. Adams, J. Jezierski, A. Luginbuhl and E. J. Bilsky (2011). "Monosodium iodoacetate-induced osteoarthritis produces pain-depressed wheel running in rats: implications for preclinical behavioral assessment of chronic pain." Pharmacology, biochemistry, and behavior **98**: 35-42.

Stirling, L. C., G. Forlani, M. D. Baker, J. N. Wood, E. A. Matthews, A. H. Dickenson and M. A. Nassar (2005). "Nociceptor-specific gene deletion using heterozygous Na V 1.8-Cre recombinase mice." *Pain* **113**(1): 27-36.

Stone, L. S., C. Broberger, L. Vulchanova, G. L. Wilcox, T. Hökfelt, M. S. Riedl and R. Elde (1998). "Differential distribution of α 2A and α 2C adrenergic receptor immunoreactivity in the rat spinal cord." *The Journal of neuroscience* **18**(15): 5928-5937.

Strassle, B., L. Mark, L. Leventhal, M. Piesla, X. J. Li, J. Kennedy, S. Glasson and G. Whiteside (2010). "Inhibition of osteoclasts prevents cartilage loss and pain in a rat model of degenerative joint disease." *Osteoarthritis and cartilage* **18**(10): 1319-1328.

Stucky, C. L. and G. R. Lewin (1999). "Isolectin B4-positive and-negative nociceptors are functionally distinct." *The Journal of neuroscience* **19**(15): 6497-6505.

Sugiura, Y., C. Lee and E. Perl (1986). "Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin." *Science* **234**(4774): 358-361.

Sullivan, A. F., M. R. Dashwood and A. H. Dickenson (1987). " α 2-Adrenoceptor modulation of nociception in rat spinal cord: location, effects and interactions with morphine." *European journal of pharmacology* **138**(2): 169-177.

Sullivan, M. D., S. Bentley, M.-Y. Fan and G. Gardner (2009). "A single-blind, placebo run-in study of duloxetine for activity-limiting osteoarthritis pain." *The journal of pain : official journal of the American Pain Society* **10**: 208-213.

Suokas, A., D. Sagar, P. Mapp, V. Chapman and D. Walsh (2014). "Design, study quality and evidence of analgesic efficacy in studies of drugs in models of OA pain: a systematic review and a meta-analysis." *Osteoarthritis and Cartilage* **22**(9): 1207-1223.

Suokas, A., D. Walsh, D. McWilliams, L. Condon, B. Moreton, V. Wylde, L. Arendt-Nielsen and W. Zhang (2012). "Quantitative sensory testing in painful osteoarthritis: a systematic review and meta-analysis." *Osteoarthritis and Cartilage* **20**(10): 1075-1085.

Suri, S., S. E. Gill, S. M. de Camin, D. F. McWilliams, D. Wilson and D. A. Walsh (2007). "Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis." *Annals of the rheumatic diseases* **66**(11): 1423-1428.

Suzuki, M., A. Mizuno, K. Kodaira and M. Imai (2003). "Impaired pressure sensation in mice lacking TRPV4." *Journal of Biological Chemistry* **278**(25): 22664-22668.

Suzuki, R., S. P. Hunt and A. H. Dickenson (2003). "The coding of noxious mechanical and thermal stimuli of deep dorsal horn neurones is attenuated in NK1 knockout mice." *Neuropharmacology* **45**(8): 1093-1100.

Suzuki, R., S. Morcuende, M. Webber, S. P. Hunt and A. H. Dickenson (2002). "Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways." *Nature neuroscience* **5**: 1319-1326.

Suzuki, R., W. Rahman, S. P. Hunt and A. H. Dickenson (2004). "Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury." *Brain research* **1019**(1): 68-76.

Suzuki, R., W. Rahman, L. J. Rygh, M. Webber, S. P. Hunt and A. H. Dickenson (2005). "Spinal-supraspinal serotonergic circuits regulating neuropathic pain and its treatment with gabapentin." *Pain* **117**: 292-303.

Suzuki, R., L. J. Rygh and A. H. Dickenson (2004). "Bad news from the brain: descending 5-HT pathways that control spinal pain processing." Trends in Pharmacological Sciences **25**(12): 613-617.

Svensson, P., K. Wang, L. Arendt-Nielsen and B. E. Cairns (2008). "Effects of NGF-induced muscle sensitization on proprioception and nociception." Experimental Brain Research **189**(1): 1-10.

Tarpley, J. W., M. G. Kohler and W. J. Martin (2004). "The behavioral and neuroanatomical effects of IB 4-saporin treatment in rat models of nociceptive and neuropathic pain." Brain research **1029**(1): 65-76.

Terayama, R., R. Dubner and K. Ren (2002). "The roles of NMDA receptor activation and nucleus reticularis gigantocellularis in the time-dependent changes in descending inhibition after inflammation." Pain **97**: 171-181.

Terayama, R., Y. Guan, R. Dubner and K. Ren (2000). "Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation." Neuroreport **11**: 1915-1919.

Thakor, D. K., A. Lin, Y. Matsuka, E. M. Meyer, S. Ruangsri, I. Nishimura and I. Spigelman (2009). "Increased peripheral nerve excitability and local Nav1. 8 mRNA up-regulation in painful neuropathy." Molecular pain **5**(1): 14.

Thakur, M. (2012). Pharmacological, Neurochemical and Functional Characterisation of the MIA Model of Experimental Osteoarthritis, University College London.

Thakur, M., J. Dawes and S. McMahon (2013). "Genomics of pain in osteoarthritis." Osteoarthritis and Cartilage **21**(9): 1374-1382.

Thakur, M., A. H. Dickenson and R. Baron (2014). "Osteoarthritis pain: nociceptive or neuropathic?" Nature Reviews Rheumatology **10**(6): 374-380.

Thakur, M., W. Rahman, C. Hobbs, A. H. Dickenson and D. L. H. Bennett (2012). "Characterisation of a peripheral neuropathic component of the rat monoiodoacetate model of osteoarthritis." PloS one **7**(3): e33730.

Thorne, C., A. D. Beaulieu, D. J. Callaghan, W. F. O'Mahony, J. M. Bartlett, R. Knight, G. R. Kraag, R. Akhras, P. S. Piraino and J. Eisenhoffer (2008). "A randomized, double-blind, crossover comparison of the efficacy and safety of oral controlled-release tramadol and placebo in patients with painful osteoarthritis." Pain Research & Management: The Journal of the Canadian Pain Society **13**(2): 93.

Todd, A. (2002). "Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor." Experimental physiology **87**(2): 245-249.

Todd, A., C. Watt, R. Spike and W. Sieghart (1996). "Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord." The Journal of neuroscience **16**(3): 974-982.

Todd, A. J. (2010). "Neuronal circuitry for pain processing in the dorsal horn." Nature Reviews Neuroscience **11**(12): 823-836.

Todd, A. J. and A. C. Sullivan (1990). "Light microscope study of the coexistence of GABA - like and glycine - like immunoreactivities in the spinal cord of the rat." Journal of Comparative Neurology **296**(3): 496-505.

Tominaga, M., M. J. Caterina, A. B. Malmberg, T. A. Rosen, H. Gilbert, K. Skinner, B. E. Raumann, A. I. Basbaum and D. Julius (1998). "The cloned capsaicin receptor integrates multiple pain-producing stimuli." Neuron **21**(3): 531-543.

Torebjörk, H., L. Lundberg and R. LaMotte (1992). "Central changes in processing of mechanoreceptive input in capsaicin - induced secondary hyperalgesia in humans." The Journal of Physiology **448**(1): 765-780.

Torsney, C. (2011). "Inflammatory pain unmasks heterosynaptic facilitation in lamina I neurokinin 1 receptor-expressing neurons in rat spinal cord." The Journal of Neuroscience **31**(13): 5158-5168.

Torsney, C. and A. B. MacDermott (2006). "Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord." The Journal of neuroscience **26**(6): 1833-1843.

Towheed, T., L. Maxwell, M. Judd, M. Catton, M. C. Hochberg and G. Wells (2006). "Acetaminophen for osteoarthritis." Cochrane Database Syst Rev **1**(1).

Treede, R.-D. and J. D. Cole (1993). "Dissociated secondary hyperalgesia in a subject with a large-fibre sensory neuropathy." Pain **53**(2): 169-174.

Tsuruoka, M., T. Hitoto, Y. Hiruma and Y. Matsui (1999). "Neurochemical evidence for inflammation-induced activation of the coeruleospinal modulation system in the rat." Brain research **821**: 236-240.

Tsuruoka, M., M. Maeda and T. Inoue (2004). "Persistent hindpaw inflammation produces coeruleospinal antinociception in the non-inflamed forepaw of rats." Neuroscience letters **367**(1): 66-70.

Tsuruoka, M., J. Tamaki, M. Maeda, B. Hayashi and T. Inoue (2012). "Biological implications of coeruleospinal inhibition of nociceptive processing in the spinal cord." Frontiers in integrative neuroscience **6**.

Tsuruoka, M. and W. D. Willis (1996). "Bilateral lesions in the area of the nucleus locus coeruleus affect the development of hyperalgesia during carrageenan-induced inflammation." Brain research **726**: 233-236.

Tsuruoka, M. and W. D. Willis (1996). "Descending modulation from the region of the locus coeruleus on nociceptive sensitivity in a rat model of inflammatory hyperalgesia." Brain Research: 86-92.

Uchimura, T., A. T. Foote, D. C. Markel, W. Ren and L. Zeng (2016). "The Chondroprotective Role of Erythromycin in a Murine Joint Destruction Model." Cartilage: 1947603516630787.

Udo, M., T. Muneta, K. Tsuji, N. Ozeki, Y. Nakagawa, T. Ohara, R. Saito, K. Yanagisawa, H. Koga and I. Sekiya (2016). "Monoiodoacetic acid induces arthritis and synovitis in rats in a dose-and time-dependent manner: proposed model-specific scoring systems." Osteoarthritis and Cartilage.

Urban, M., S. Coutinho and G. Gebhart (1999). "Involvement of excitatory amino acid receptors and nitric oxide in the rostral ventromedial medulla in modulating secondary hyperalgesia produced by mustard oil." Pain **81**(1-2): 45.

Urban, M., M. Jiang and G. Gebhart (1996). "Participation of central descending nociceptive facilitatory systems in secondary hyperalgesia produced by mustard oil." Brain Research **737**(1-2): 83-91.

Urban, M., P. Zahn and G. Gebhart (1999). "Descending facilitatory influences from the rostral medial medulla mediate secondary, but not primary hyperalgesia in the rat." Neuroscience **90**(2): 349-352.

Urban, M. O. and G. F. Gebhart (1999). "Supraspinal contributions to hyperalgesia." Proceedings of the National Academy of Sciences of the United States of America **96**: 7687-7692.

Urch, C. and A. Dickenson (2003). "In vivo single unit extracellular recordings from spinal cord neurones of rats." Brain research protocols **12**(1): 26-34.

Uta, D., H. Furue, A. E. Pickering, M. Harunor Rashid, H. Mizuguchi - Takase, T. Katafuchi, K. Imoto and M. Yoshimura (2010). "TRPA1 - expressing primary afferents synapse with a morphologically identified subclass of substantia gelatinosa neurons in the adult rat spinal cord." European Journal of Neuroscience **31**(11): 1960-1973.

Valdes, A. M. and T. D. Spector (2010). "The genetic epidemiology of osteoarthritis." Current opinion in rheumatology **22**(2): 139-143.

van der Kraan, P. M., E. L. Vitters, L. Van de Putte and W. B. van den Berg (1989). "Development of osteoarthritic lesions in mice by " metabolic" and " mechanical" alterations in the knee joints." The American journal of pathology **135**(6): 1001.

Van Osch, G., P. Van Der Kraan and W. Van Den Berg (1994). "Site - specific cartilage changes in murine degenerative knee joint disease induced by iodoacetate and collagenase." Journal of orthopaedic research **12**(2): 168-175.

Vanegas, H. (2004). "To the descending pain-control system in rats, inflammation-induced primary and secondary hyperalgesia are two different things." Neuroscience letters **361**: 225-228.

Vanegas, H. and H.-G. Schaible (2004). "Descending control of persistent pain: inhibitory or facilitatory?" Brain research. Brain research reviews **46**: 295-309.

Vasko, M. (1995). "Prostaglandin-induced neuropeptide release from spinal cord." Progress in Brain Research **104**: 367-380.

Vasko, M. R., I.-H. Pang and M. Vogt (1984). "Involvement of 5-hydroxytryptamine-containing neurons in antinociception produced by injection of morphine into nucleus raphe magnus or onto spinal cord." Brain research **306**(1): 341-348.

Vecchiet, L., J. Vecchiet and M. A. Giamberardino (1999). "Referred muscle pain: clinical and pathophysiologic aspects." Current pain and headache reports **3**(6): 489-498.

Vedula, S. S., L. Bero, R. W. Scherer and K. Dickersin (2009). "Outcome reporting in industry-sponsored trials of gabapentin for off-label use." New England Journal of Medicine **361**(20): 1963-1971.

Vera-Portocarrero, L. P., J. X. Yie, J. Kowal, M. H. Ossipov, T. King and F. Porreca (2006). "Descending facilitation from the rostral ventromedial medulla maintains visceral pain in rats with experimental pancreatitis." Gastroenterology **130**(7): 2155-2164.

Vignon, E., J. Balblanc, P. Mathieu, P. Louisot and M. Richard (1993). "Metalloprotease activity, phospholipase A2 activity and cytokine concentration in osteoarthritis synovial fluids." Osteoarthritis and Cartilage **1**(2): 115-120.

Vijayaragavan, K., M. Boutjdir and M. Chahine (2004). "Modulation of Nav1. 7 and Nav1. 8 peripheral nerve sodium channels by protein kinase A and protein kinase C." Journal of Neurophysiology **91**(4): 1556-1569.

Vilceanu, D. and C. L. Stucky (2010). "TRPA1 mediates mechanical currents in the plasma membrane of mouse sensory neurons." PloS one **5**(8): e12177.

Vonsy, J. L. (2008). The monosodium iodoacetate induced model of osteoarthritis pain: behavioural, pharmacological, immunohistochemical and electrophysiological studies, UCL.

Vonsy, J. L., J. Ghandehari and A. H. Dickenson (2009). "Differential analgesic effects of morphine and gabapentin on behavioural measures of pain and disability in a model of osteoarthritis pain in rats." European journal of pain (London, England) **13**: 786-793.

Vrontou, S., A. M. Wong, K. K. Rau, H. R. Koerber and D. J. Anderson (2013). "Genetic identification of C fibres that detect massage-like stroking of hairy skin in vivo." *Nature* **493**(7434): 669-673.

Vulchanova, L., T. Olson, L. Stone, M. Riedl, R. Elde and C. Honda (2001). "Cytotoxic targeting of isolectin IB4-binding sensory neurons." *Neuroscience* **108**(1): 143-155.

Walsh, D. (1999). "Angiogenesis and arthritis." *Rheumatology* **38**(2): 103-112.

Walsh, D., C. Bonnet, E. Turner, D. Wilson, M. Situ and D. McWilliams (2007). "Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis1." *Osteoarthritis and Cartilage* **15**(7): 743-751.

Walsh, D. A., D. F. McWilliams, M. J. Turley, M. R. Dixon, R. E. Fransès, P. I. Mapp and D. Wilson (2010). "Angiogenesis and nerve growth factor at the osteochondral junction in rheumatoid arthritis and osteoarthritis." *Rheumatology* **49**(10): 1852-1861.

Wang, R., T. King, M. De Felice, W. Guo, M. H. Ossipov and F. Porreca (2013). "Descending facilitation maintains long-term spontaneous neuropathic pain." *The Journal of Pain* **14**(8): 845-853.

Wang, Y., J. Wu, Z. Wu, Q. Lin, Y. Yue and L. Fang (2010). "Regulation of AMPA receptors in spinal nociception." *Molecular Pain* **6**(1): 5.

Waters, A. J. and B. M. Lumb (2008). "Descending control of spinal nociception from the periaqueductal grey distinguishes between neurons with and without C-fibre inputs." *Pain* **134**(1): 32-40.

Watson, A. H., D. I. Hughes and A. A. Bazzaz (2002). "Synaptic relationships between hair follicle afferents and neurones expressing GABA and glycine - like immunoreactivity in the spinal cord of the rat." *Journal of Comparative Neurology* **452**(4): 367-380.

Wei, F., R. Dubner and K. Ren (1999). "Nucleus reticularis gigantocellularis and nucleus raphe magnus in the brain stem exert opposite effects on behavioral hyperalgesia and spinal Fos protein expression after peripheral inflammation." *Pain* **80**: 127-141.

Wei, F., R. Dubner, S. Zou, K. Ren, G. Bai, D. Wei and W. Guo (2010). "Molecular depletion of descending serotonin unmasks its novel facilitatory role in the development of persistent pain." *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**: 8624-8636.

Weiss, J., M. Pyrski, E. Jacobi, B. Bufe, V. Willnecker, B. Schick, P. Zizzari, S. J. Gossage, C. A. Greer and T. Leinders-Zufall (2011). "Loss-of-function mutations in sodium channel Nav1. 7 cause anosmia." *Nature* **472**(7342): 186-190.

West, W. L., D. C. Yeomans and H. K. Proudfit (1993). "The function of noradrenergic neurons in mediating antinociception induced by electrical stimulation of the locus coeruleus in two different sources of Sprague-Dawley rats." *Brain research* **626**(1): 127-135.

Westlund, K., R. Bowker, M. Ziegler and J. Coulter (1983). "Noradrenergic projections to the spinal cord of the rat." *Brain research* **263**(1): 15-31.

Westlund, K. and A. Craig (1996). "Association of spinal lamina I projections with brainstem catecholamine neurons in the monkey." *Experimental brain research* **110**(2): 151-162.

Westlund, K. N., R. M. Bowker, M. G. Ziegler and J. D. Coulter (1984). "Origins and terminations of descending noradrenergic projections to the spinal cord of monkey." *Brain Research* **292**(1): 1-16.

Whiteside, G., J. Dwyer, J. Harrison, C. Beyer, T. Cummons, L. Manzano, L. Mark, G. Johnston, B. Strassle and A. Adedoyin (2010). "WAY - 318068: a novel, potent and selective noradrenaline re - uptake inhibitor with activity in rodent models of pain and depression." British journal of pharmacology **160**(5): 1105-1118.

Wiberg, M., J. Westman and A. Blomqvist (1987). "Somatosensory projection to the mesencephalon: an anatomical study in the monkey." Journal of Comparative Neurology **264**(1): 92-117.

Widenfalk, B. and M. Wiberg (1989). "Origin of sympathetic and sensory innervation of the knee joint." Anatomy and embryology **180**(4): 317-323.

Wieland, H. A., M. Michaelis, B. J. Kirschbaum and K. A. Rudolphi (2005). "Osteoarthritis,Âan untreatable disease?" Nature Reviews Drug Discovery **4**(4): 331-344.

Willis, W., D. Kenshalo and R. Leonard (1979). "The cells of origin of the primate spinothalamic tract." Journal of comparative neurology **188**(4): 543-573.

Willis, W. D. (1985). The pain system, Karger Stockholm.

Winkler, C. W., S. M. Hermes, C. I. Chavkin, C. T. Drake, S. F. Morrison and S. A. Aicher (2006). "Kappa opioid receptor (KOR) and GAD67 immunoreactivity are found in OFF and NEUTRAL cells in the rostral ventromedial medulla." Journal of neurophysiology **96**(6): 3465-3473.

Wood, J. N., J. P. Boorman, K. Okuse and M. D. Baker (2004). "Voltage - gated sodium channels and pain pathways." Journal of neurobiology **61**(1): 55-71.

Woolf, C. and M. Salter (2006). "Plasticity and pain: role of the dorsal horn." Wall and Melzack's textbook of pain **5**: 91-105.

Woolf, C., S. Thompson and A. King (1988). "Prolonged primary afferent induced alterations in dorsal horn neurones, an intracellular analysis in vivo and in vitro." Journal de physiologie **83**(3): 255.

Woolf, C. J. (2004). "Pain : Moving from Symptom Control toward Mechanism-Specific Pharmacologic Management." Annals of Internal Medicine **140**: 441-451.

Woolf, C. J. (2011). "Central sensitization: implications for the diagnosis and treatment of pain." Pain **152**: S2-15.

Wylde, V., S. Hewlett, I. D. Learmonth and P. Dieppe (2011). "Persistent pain after joint replacement: Prevalence, sensory qualities, and postoperative determinants." Pain **152**(3): 566-572.

Wylde, V., S. Palmer, I. D. Learmonth and P. Dieppe (2012). "Somatosensory abnormalities in knee OA." Rheumatology **51**(3): 535-543.

Xiao, R. and X. Xu (2010). "Mechanosensitive channels: in touch with Piezo." Current Biology **20**(21): R936-R938.

Yanarates, O., A. Dogrul, V. Yildirim, A. Sahin, A. Sizlan, M. Seyrek, Ö. Akgül, O. Kozak, E. Kurt and U. Aypar (2010). "Spinal 5-HT₇ receptors play an important role in the antinociceptive and antihyperalgesic effects of tramadol and its metabolite, O-desmethyltramadol, via activation of descending serotonergic pathways." Anesthesiology **112**(3): 696-710.

Yang, Y., Y. Wang, S. Li, Z. Xu, H. Li, L. Ma, J. Fan, D. Bu, B. Liu and Z. Fan (2004). "Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythromalgia." Journal of medical genetics **41**(3): 171-174.

Yasaka, T., G. Kato, H. Furue, M. H. Rashid, M. Sonohata, A. Tamae, Y. Murata, S. Masuko and M. Yoshimura (2007). "Cell - type - specific excitatory and inhibitory circuits involving primary afferents in the substantia gelatinosa of the rat spinal dorsal horn in vitro." The Journal of physiology **581**(2): 603-618.

Yelin, E., D. Lubeck, H. Holman and W. Epstein (1987). "The impact of rheumatoid arthritis and osteoarthritis: the activities of patients with rheumatoid arthritis and osteoarthritis compared to controls." The Journal of rheumatology **14**(4): 710-717.

Yoon, J. S., S. J. Won, S. J. Kim, S. J. Hong, G. S. Kim and J. M. Kim (2015). "Phase-Contrast Radiography Enables Detection of Early Changes in Articular Cartilage in a Mouse Model of Osteoarthritis." American Journal of Physical Medicine & Rehabilitation **94**(8): 644-648.

Yoon, Y. W., D. H. Lee, B. H. Lee, K. Chung and J. M. Chung (1999). "Different strains and substrains of rats show different levels of neuropathic pain behaviors." Experimental brain research **129**(2): 167-171.

Yoshimi, E., H. Yamamoto, Y. Furuichi, Y. Shimizu and N. Takeshita (2010). "Sustained analgesic effect of the Rho kinase inhibitor AS1892802 in rat models of chronic pain." Journal of pharmacological sciences **114**(1): 119-122.

Young, R. F. and T. Brechner (1986). "Electrical stimulation of the brain for relief of intractable pain due to cancer." Cancer **57**(6): 1266-1272.

Zagon, A. and S. Bacon (1991). "Evidence of a monosynaptic pathway between cells of the ventromedial medulla and the motoneuron pool of the thoracic spinal cord in rat: electron microscopic analysis of synaptic contacts." European Journal of Neuroscience **3**(1): 55-65.

Zakrzewska, J. M., J. Palmer, D. A. Ettlin, M. Obermann, G. M. Giblin, V. Morisset, S. Tate and K. Gunn (2013). "Novel design for a phase IIa placebo-controlled, double-blind randomized withdrawal study to evaluate the safety and efficacy of CNV1014802 in patients with trigeminal neuralgia." Trials **14**(1): 402.

Zambreanu, L., R. Wise, J. Brooks, G. Iannetti and I. Tracey (2005). "A role for the brainstem in central sensitisation in humans. Evidence from functional magnetic resonance imaging." Pain **114**(3): 397-407.

Zavan, B., L. Ferroni, C. Giorgi, G. Calò, P. Brun, R. Cortivo, G. Abatangelo and P. Pinton (2013). "Hyaluronic Acid Induces Activation of the κ -Opioid Receptor." PLOS ONE **8**(1): e55510.

Zeitz, K. P., N. Guy, A. B. Malmberg, S. Dirajlal, W. J. Martin, L. Sun, D. W. Bonhaus, C. L. Stucky, D. Julius and A. I. Basbaum (2002). "The 5-HT₃ subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors." The Journal of neuroscience **22**(3): 1010-1019.

Zeitz, K. P., N. Guy, A. B. Malmberg, S. Dirajlal, W. J. Martin, L. Sun, D. W. Bonhaus, C. L. Stucky, D. Julius and A. I. Basbaum (2002). "The 5-HT₃ Subtype of Serotonin Receptor Contributes to Nociceptive Processing via a Novel Subset of Myelinated and Unmyelinated Nociceptors." In Situ **22**: 1010-1019.

Zeni, J. A. and J. S. Higginson (2011). "Knee osteoarthritis affects the distribution of joint moments during gait." The Knee **18**(3): 156-159.

Zhang, J., D. J. Cavanaugh, M. I. Nemenov and A. I. Basbaum (2013). "The modality - specific contribution of peptidergic and non - peptidergic nociceptors is manifest at the level of dorsal horn nociresponsive neurons." The Journal of physiology **591**(4): 1097-1110.

Zhang, L. and D. L. Hammond (2010). "Cellular basis for opioid potentiation in the rostral ventromedial medulla of rats with persistent inflammatory nociception." Pain **149**: 107-116.

Zhang, L., A. O. Hoff, S. J. Wimalawansa, G. J. Cote, R. F. Gagel and K. N. Westlund (2001). "Arthritic calcitonin/ α calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity." *Pain* **89**(2): 265-273.

Zhang, W., M. Doherty, N. Arden, B. Bannwarth, J. Bijlsma, K.-P. Gunther, H. J. Hauselmann, G. Herrero-Beaumont, K. Jordan and P. Kaklamanis (2005). "EULAR evidence based recommendations for the management of hip osteoarthritis: report of a task force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT)." *Annals of the rheumatic diseases* **64**(5): 669-681.

Zhang, W., A. Jones and M. Doherty (2004). "Does paracetamol (acetaminophen) reduce the pain of osteoarthritis?: a meta-analysis of randomised controlled trials." *Annals of the rheumatic diseases* **63**(8): 901-907.

Zhang, W., R. Moskowitz, G. Nuki, S. Abramson, R. Altman, N. Arden, S. Bierma-Zeinstra, K. Brandt, P. Croft and M. Doherty (2007). "OARSI recommendations for the management of hip and knee osteoarthritis, part I: critical appraisal of existing treatment guidelines and systematic review of current research evidence." *Osteoarthritis and Cartilage* **15**(9): 981-1000.

Zhang, W., R. Moskowitz, G. Nuki, S. Abramson, R. Altman, N. Arden, S. Bierma-Zeinstra, K. Brandt, P. Croft and M. Doherty (2008). "OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines." *Osteoarthritis and cartilage* **16**(2): 137-162.

Zhang, W., G. Nuki, R. Moskowitz, S. Abramson, R. Altman, N. Arden, S. Bierma-Zeinstra, K. Brandt, P. Croft and M. Doherty (2010). "OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009." *Osteoarthritis and Cartilage* **18**(4): 476-499.

Zhang, Y., L. Xu, M. C. Nevitt, P. Aliabadi, W. Yu, M. Qin, L. Y. Lui and D. T. Felson (2001). "Comparison of the prevalence of knee osteoarthritis between the elderly Chinese population in Beijing and whites in the United States: The Beijing Osteoarthritis Study." *Arthritis & Rheumatism* **44**(9): 2065-2071.

Zhang, Y., L. Xu, M. C. Nevitt, J. Niu, J. P. Goggins, P. Aliabadi, W. Yu, L. Y. Lui and D. T. Felson (2003). "Lower prevalence of hand osteoarthritis among Chinese subjects in Beijing compared with white subjects in the United States: the Beijing Osteoarthritis Study." *Arthritis & Rheumatism* **48**(4): 1034-1040.

Zhang, Y., S. Zhao, E. Rodriguez, J. Takatoh, B.-X. Han, X. Zhou and F. Wang (2015). "Identifying local and descending inputs for primary sensory neurons." *The Journal of clinical investigation* **125**(10): 3782.

Zhuo, M. and G. Gebhart (1990). "Characterization of descending inhibition and facilitation from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat." *Pain* **42**(3): 337-350.

Zhuo, M. and G. Gebhart (1991). "Spinal serotonin receptors mediate descending facilitation of a nociceptive reflex from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat." *Brain research* **550**(1): 35-48.

Zhuo, M. and G. Gebhart (1992). "Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat." *Journal of Neurophysiology* **67**(6): 1599-1614.

- Zhuo, M. and G. Gebhart (1997). "Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat." Journal of Neurophysiology **78**(2): 746.
- Ziegler, E., W. Magerl, R. Meyer and R.-D. Treede (1999). "Secondary hyperalgesia to punctate mechanical stimuli." Brain **122**(12): 2245-2257.
- Zimmermann, K., A. Leffler, A. Babes, C. M. Cendan, R. W. Carr, J. Kobayashi, C. Nau, J. N. Wood and P. W. Reeh (2007). "Sensory neuron sodium channel Nav1. 8 is essential for pain at low temperatures." Nature **447**(7146): 856-859.
- Zimmermann, M. (1983). "Ethical guidelines for investigations of experimental pain in conscious animals." Pain **16**(2): 109-110.
- Zou, X., Q. Lin and W. D. Willis (2000). "Enhanced phosphorylation of NMDA receptor 1 subunits in spinal cord dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats." The Journal of neuroscience **20**(18): 6989-6997.
- Zylka, M. J., F. L. Rice and D. J. Anderson (2005). "Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd." Neuron **45**(1): 17-25.
- Zylka, M. J., N. A. Sowa, B. Taylor-Blake, M. A. Twomey, A. Herrala, V. Voikar and P. Vihko (2008). "Prostatic acid phosphatase is an ectonucleotidase and suppresses pain by generating adenosine." Neuron **60**(1): 111-122.